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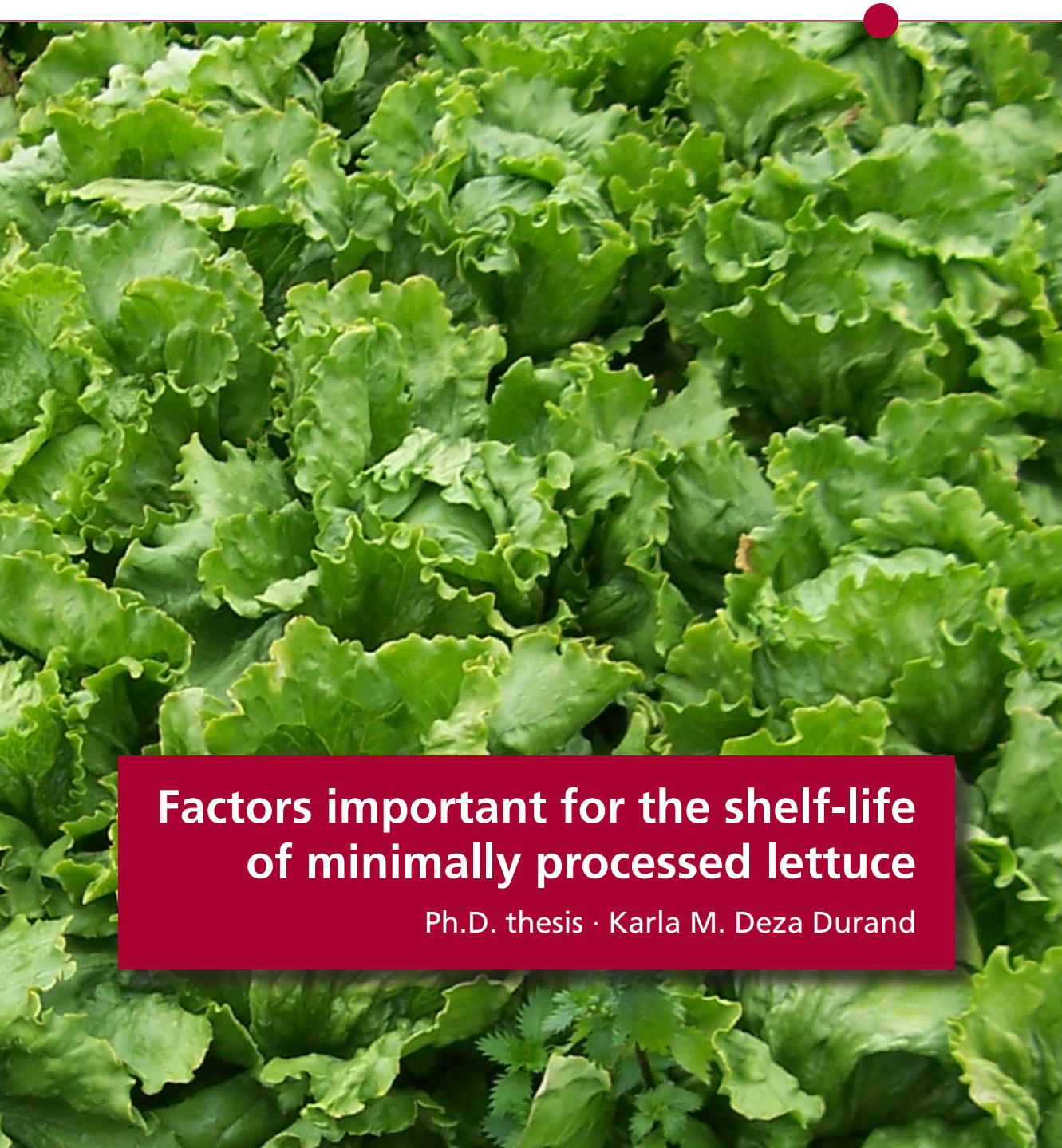

Factors important for the shelf-life of minimally processed lettuce

Ph.D. thesis · Karla M. Deza Durand

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Ph.D. thesis

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Preface

This thesis is part of the requirement for obtaining a PhD degree at University of Copenhagen. The PhD was founded by the University of Copenhagen through a scholarship. The work was carried out in the facilities of the Quality and Technology group, Department of Food Science at the Faculty of Science. This project also involved GASA Odense, Rijk Zwaan, Bladgrønt and Bemis as discussion partners and suppliers of raw materials and MAP products. The lettuce used in the experiments were kindly donated by Rijk Zwaan and sowed in the commercial facilities of Bladgrønt. I would like to thank Mogen and Ulla Mogense for providing me part of their lettuce field to grow the lettuce used in this project and Gasa Odense for processing and packaging the plant material. Additionally, to Bemis for supplying the film packages.

I would like to thank especially to Mikael Agerlin Petersen for his guidance as supervisor and for sharing with me his knowledge about aroma, and to Abdel and to the late Mehdi D. Farahani for their technical assistance during my research. I would to thank to all the people of quality and technology group, especially to Margaret Owusu my friend and former officemate and to Marta and Camilla, for the nice working atmosphere and friendship.

Finally, to my husband, thanks for your patience and support and to my son Erik, who always draw a smile in my face and for reminding me what is important in life. Above all, I thank God for giving me the opportunity to work in this PhD project.

Karla M. Deza Durand

January 2013

Summary

The minimally processed vegetable industry has been increasing rapidly due to change in lifestyle. Both women and men work outside home and have less time to cook and need more convenience and time saving products, which also present fresh and healthy characteristics. Iceberg lettuce (*Lactuca sativa* L.) is one of the most popular fresh-cut vegetables. Although an increase in the number of mixed salads in retail food chains is evident, their short shelf-life due to rapid browning and off-odour is a problem that need research. Therefore, the aim of this PhD project was to investigate factors important for the shelf-life of minimally processed iceberg lettuce and to propose a new methodology to measure browning in cut lettuce.

Browning has been pointed out as the main factor limitating shelf-life in cut lettuce. The problem becomes complex because browning of cut lettuce is difficult to measure. A novel method using image analysis for the measurement of browning in minimally processed lettuce was developed and presented in **paper I**. The method used a flatbed scanner for image acquisition, colour dye patches for colour correction, and colour thresholding to quantify the browning, that was expressed as brown area fraction. Cut lettuce was stored at 5°C for 6 days and plus 1 day at room temperature (day 7). Changes in browning were assessed at 2, 6 and 7 days of storage using image analysis. The result showed an increase in browning as time and temperature of storage increased. It was concluded that this technique can be used for measuring the browning in cut lettuce.

Few studies are done on the formation of volatiles in cut lettuce. Temperature of storage and methods of preparation that minimized quality loss are highly desirable. As such, cutting direction and storage temperature were investigated to elucidate their influence on aroma formation and respiration rate in minimally processed lettuce, are presented in **paper II**. Lettuce was cut longitudinal and transverse to the mid-rib and stored at 6 and 10°C for 4 and 5 days. Changes in respiration rate were analyzed through the storage time, and aroma analysis was carried out after 4 and 5 days of storage in January and

March, respectively. Respiration rate increased with increasing storage temperatures. Aroma formation was also influenced by storage temperature. Higher storage temperature allowed the increase of α-longipinene, 2-methylbutanal and 3-methylbutanal. Transversal cut to the rib was strongly related with volatiles of lipoxygenase (LOX) pathway i.e. cis-3-hexenal, cis-3-hexenol and trans-2-hexenol, meanwhile longitudinal cut enhanced the formation of volatiles from other metabolic routes. Therefore, it was concluded that transversal cut cause a more severe damage to the tissue than longitudinal cut based on aroma production of LOX volatiles.

It has been indicated that cultivar, season, packaging and storage time influence the type and concentration of volatile compounds, browning, chemical constituents and texture in vegetables. As part of this project, a more integrated study was undertaken for first time in lettuce to our knowledge. The study took into account the influence of cultivar, season, packaging and storage time. In order to achieve this, iceberg lettuce cultivars Platinas, Diamantinas and Morinas were harvested from June to September 2009. Lettuces were minimally processed and stored under three different treatments: two passive modified atmosphere packaging (MAP) built up by films of different permeabilities, F1 (OPALEN 65 AF) and F2 (OPP/PE-L 2040 AF), and storage in air. All packages were stored at 5°C. Gas composition, volatile compounds, physicochemical constituents, PPO activity and browning were assessed at 1, 5, 8 and 11 days of storage in packaged lettuce, whereas in air stored samples only at 1 and 5 days of storage. Additionally, respiration rate was only assessed in air stored samples and GC-O analysis was undertaken after 1 and 11 days of storage for cultivar Morinas packaged in passive MAP F2 in September. The study was divided into three papers, **paper III, IV** and **paper V.** The study in **paper III** was limited to analyze the volatiles compounds as a function of packaging and storage time and was used as basis for a more comprehensive analyses as presented in **paper IV**.

Paper III revealed that packaging and storage time had an influence on the volatiles of cut iceberg lettuce allowing the formation of desirable aroma but also on the development of off-odors. This result indicates that extremely low O_2 and high CO_2 conditions found

in the passive MAP F1 and F2 after 11 days of storage enhanced the formation of volatiles of anaerobic metabolism such as ethyl acetate and 2,3-butanedione.

In paper IV 52 volatile compounds were identified and of these 21 potent odorants were shown to contribute to the aroma of cut lettuce. Among them elemene, caryophyllene, β-selinene and 2,3-butanedione, enhanced under anaerobic conditions and likely to be off-odours. In August high production of these odorants probably compromised the quality in terms of odour. The findings suggest that most of the potent odorants enhanced their relative area under anaerobic conditions built up in the passive MAPs during the storage time, and are likely to produce off-odour. Levels of odorants such as 2,3-butanedione, elemene, caryophyllene and β-selinene were significantly enhanced under anaerobic conditions after 11 days of storage, being significantly higher in passive MAP F1. Regarding the cultivars, Morinas and Diamantinas was the less tolerant to high CO_2 resulting in significantly higher amount of 2,3-butanedione.

P**aper V** was related to browning and other physicochemical characteristics of minimally processed lettuce such as soluble sugars, organic acids, chlorogenic acid, pH, polyphenol oxidase activity and firmness. Gas chromatography mass spectrometry (GC-MS) method was developed for the analysis of soluble sugars, organic acids and chlorogenic acid in cut lettuce. Our results showed that season and storage time mainly influence over physicochemical characteristics of packaged and air stored cut lettuce, and in less degree the cultivar. In June, fructose, glucose, sucrose, malic acid and firmness were kept high under anaerobic conditions. Differences in the content of O_2 and CO_2 between the passive MAPs and air stored samples demonstrated to influence the formation of browning and other physicochemical characteristics as storage time increased. It was concluded that browning was remarkable controlled in passive MAPs samples, irrespective of season and cultivar due to extremely low O_2 and high CO_2 conditions, however, after 11 days of storage, this condition favored tissue softening, decreased of sugars and malolactic fermentation, mainly in passive MAP F1.

List of publications

Paper I: Deza-Durand, K.M., Petersen, M.A., Poll, L., Larsen, M. 2009. Image analysis for measuring enzymatic browning in minimally processed lettuce, in: Sørensen, H., Sørensen, S., Sørensen, A.D., Sørensen, J.C., Andersen, K.E., Bjergegaard, C., Møller, P. (Eds.), Euro Food Chem XV-FOOD FOR THE FUTURE-the contribution of chemistry to improvement of food quality. Book I of Proceedings. Faculty of Life Science, University of Copenhagen, Denmark, pp.111-114.

Paper II: Deza-Durand, K.M., Petersen, M.A. 2011. The effect of cutting direction on aroma compounds and respiration rate of fresh-cut iceberg lettuce (*Lactuca sativa* L.) Postharvest Biology and Technology, volume 61, issue 1, pp. 83-90.

Paper III: Deza-Durand, K.M., Petersen, M.A., Roepstorff, M., Poll, L. 2011. Influence of packaging and storage time on aroma compounds of minimally processed lettuce, in: Hofmann, T., Meyerhof, W., Schieberle, P. (Eds.), Advances and challenges in flavor chemistry and biology. Proceedings of the 9th Wartburg Symposium. DFA, Germany, pp. 305-309.

Paper IV: Deza-Durand, K.M., Petersen, M.A. 2013. Effect of season, cultivar, packaging and storage time on volatile formation of cut iceberg lettuce. Postharvest Biology and Technology (submitted).

Paper V: Deza-Durand, K.M., Petersen, M.A. 2013. Changes in physicochemical characteristics of packaged and air stored cut iceberg lettuce upon storage and season (manuscript under preparation).

Table of Contents

Chapter 1. Introduction

Cut Iceberg lettuce (*Lactuca sativa* L.) is considered one of the most popular of minimally processed vegetables, and a growing demand for this convenient and time saving product has occurred in developed countries over the last few years (Ragaert et al., 2004). This vegetable is very popular due to its crispy texture, attractive green leaves, neutral taste and green aroma (Rico et al., 2007).

During cutting the disruption of the cell induces numerous physiological changes such as an increase in the respiration and mixing of phenolic acids and polyphenol oxidase, which causes browning (Couture et al., 1993; Saltveit, 2003). Tissue disruption can also initiate the formation of volatiles, like C6 aldehydes from LOX pathway. These volatiles are important odorants of lettuce, but in higher concentration can become off-odours. Important factors that influence the browning and volatiles in fruits and vegetables are season, cultivar, method of preparation, packaging, storage temperature and storage time, among others (Matheis et al., 1983; Heimdal et al., 1995; Smyth et al., 1998; Fukumoto et al., 2004; Hodges and Toivonen, 2008; Cantos et al., 2001).

Volatile products of cut lettuce have received little attention, even though they are important parameters in assessing its quality (Smyth et al., 1998; Nielsen and Poll, 2006; Lonchamp et al., 2009). Methods of preparation of cut lettuce that minimize cutting damage are highly desirable. There are some volatiles that are more specific to mechanical injury such as C6 aldehydes from LOX pathway, which can be used as indicators of severe cutting damage. Furthermore, the influence of different storage temperatures related to the development of volatiles in cut lettuce is unknown, but in packaged broccoli stored at 10 ºC volatile compounds increased their concentration in comparison with broccolis stored at 4 ºC (Jacobsson et al., 2004).

Modified atmosphere packaging has showed to be effective to extend the shelf-life of fresh-cut lettuce by reducing browning (Bolin and Huxsoll, 1991; Heimdal et al., 1995). However, off-odours can be developed due to extremely low O_2 and high CO_2 levels build up in the package. Therefore, off-odour can be a serious limiting factor for the quality and marketability of cut lettuce.

Fresh cut lettuce industry in Denmark uses different cultivars of lettuce depending on availability during season. Thus the problem becomes complex because large cultivar variation could implicate changes in the rate of enzymatic browning and in both quantitative and qualitative formation of off-odours (Forney et al., 2000). Likewise, season could affect the volatile formation in lettuce. For example in *Brassica* specie, the changes of sulfur volatiles within a season are caused by variations in the amount of aroma precursors, i.e. glucosinolates, as a result of changes in environmental conditions (Mattheis and Fellman, 1999; Vallejo et al., 2003).

Furthermore, objective measurements of browning in cut lettuce have been made using colorimeters (Heimdal et al., 1995), however, it has been claimed to be less successful to obtain an accurate colour representation due to point by point measurements (O'Sullivan et al., 2003). Under this context, other methodology like image analysis is a prominent choice, which has been reported to be representative, consistent and cost effective (O'Sullivan et al., 2003).

Aim of the thesis

The overall objective of this PhD thesis was to generate more knowledge about the formation of volatiles, browning and other physicochemical characteristics such as glucose, fructose, sucrose, malic acid, tartaric acid, ascorbic acid, chlorogenic acid, pH, polyphenol oxidase activity and firmness as a function of season, package, method of preparation, temperature of storage and storage time. The specific objectives of this research were:

- To investigate the aroma compounds and respiration rate of cut lettuce as a function of cutting direction and storage temperature (**paper II**).
- To investigate the changes in aroma compounds of cut lettuce as a function of season, cultivar, package and storage time (**paper III and IV**).
- To develop a new technique to assess the browning of cut lettuce (**paper I**)
- To investigate the changes of browning and other physicochemical characteristics of cut lettuce as a function of season, cultivar, package and storage time (**paper V**).

The results of this PhD thesis have been published in peer reviewed journals (paper II) and in conference proceedings (paper I and III) and submitted to peer reviewed journals (paper IV). Paper V is still under preparation.

Thesis outline

The thesis is divided into seven chapters. A brief description is given below.

Chapter 1. *Introduction*, gives an introduction to the problems associated to minimally processed iceberg lettuce. It also presents the scope of the thesis and lists the specific objectives.

Chapters 2. *The lettuce*, provides an overview of iceberg lettuce, e.g. taxonomy, cultivation and respiration rate.

Chapter 3. *Minimally processed lettuce*, describes each step of the processing and its influence on browning and aroma of lettuce.

Chapter 4. *Aroma of minimally processed lettuce*, provides a definition of aroma and describes the possible metabolic routes for the formation of volatiles in lettuce. It also includes an introduction to the analytical methods used for the analysis of volatiles e.g. dynamic headspace sampling, GC-MS and GC-O. Effect of important factors on the formation of volatiles in cut lettuce is provided through of paper II, III and IV.

Chapter 5. *Enzymatic browning and other physicochemical characteristic of minimally processed lettuce*, gives an overview of the process of browning and methodology for its measurement e.g. image analysis (paper I). It also provides theoretical background for measurement of texture in lettuce and introduction to the analysis of sugar, acids,

ascorbic acid and chlorogenic acids using GC-MS. Paper V is used to explain the effect of important factors on browning and physicochemical properties of lettuce.
Chapter 6 and provides *conclusion* and *perspectives* of the PhD research.

Chapter 2. The lettuce

2.1. Taxonomy

The specie *Lactuca sativa* comprises seven main groups of cultivars: Butterhead lettuce, Iceberg or crisphead lettuce, Romaine/Cos lettuce, Cutting lettuce, Stalk lettuce, Latin lettuce and Oilseed lettuce. Among them, iceberg lettuce is the most popular minimally processed leafy vegetable. Its consumption has increased dramatically in recent years due to consumers needing more convenience and time saving products, which also present fresh and healthy characteristics (Ragaert et al., 2004; Rico et al., 2007).

Table 1. Classification of Iceberg lettuce

Taxonomic hierarchy	*Latin name*
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Asterales
Family	Asteraceae
Genus	Lactuca L.
Specie	*Lactuca sativa* L.

(http://plants.usda.gov/java/ClassificationServlet?source=profile&symbol=LACTU&display=63)

2.2. Anatomy

Lettuce leave is made of vascular and photosynthetic tissue (Toole et al., 2000), where a thick white mid-rib (vascular tissue) constitutes the majority of the leaf (photosynthetic tissue). The leaves are tightly wrapped and interlocked providing a crispy texture to lettuce (Fig.1) (de Vries, 1997; Toole et al; 2000; Křístková et al. 2008). Previous works indicate that photosynthetic and vascular tissues possess different phenolic metabolism as well as textural characteristics (Toole et al; 2000). Vascular tissue has lower polyphenol oxidase, peroxidase and phenylamonium lyase activity than photosynthetic tissue

(Heimdal et al., 1995; Fukumoto et al., 2002). However the potential for browning development is higher in vascular tissue, mainly in outer than inner leaves probably due to vascular tissue having a higher total volume and cut area (Fukumoto et al., 2002). On the other hand, there is no information, to our knowledge, regarding differences in the formation of volatiles between both tissues. In **paper II** it is hypothesized that the differences in the formation of volatiles between transversal and longitudinal cutting could be related to increase cutting surface area and damage to more cells of the mid-rib (vascular tissue) which enhanced the formation of LOX volatiles. Further research is needed in this area to understand volatile formation in fresh-cut lettuce.

a) Whole head (outside)

b) Cut in half (inside view)

Figure 1. Iceberg lettuce (*Lactuca sativa* L.)

2.3. Cultivation

Iceberg lettuce is produced commercially in North and Central America, Asia and Europe (Mou, 2008). In Denmark, the season of growing lettuce begins in April and ends in September. The rest of the year lettuce is imported mainly from Spain and Holland to supply the market (Gasa, oral communication). Field production of iceberg lettuce is carried out using transplanting seedlings (Fig.2) (Kristensen et al., 1987). Seedlings are planted from April to July. In June and September lettuce takes approximately 8 weeks to

grow to commercial maturity and in July and August it takes 6 to 7 weeks. Lettuces are harvested manually at commercial maturity when the head is well formed, compact and has a weight in the range from 400 to 500g (personal communication with the growers). Commercial maturity is defined as the stage of development when a plant or plant part has characteristics for an economical utilization for a particular purpose by the consumer (Shewfelt, 1986). Head firmness under hand pressure is used for classification of maturity (Kader et al., 1973). It is important to mention that in this manuscript and in the **papers II** to **V** the term "maturity" refers to "commercial maturity".

Several cultivars of iceberg lettuce are available for cultivation in Denmark (Rijk Zwaan catalog 2007). Growers choose a cultivar based mainly on is resistance against tipburn and other diseases, well formed head and good speed for early production. However a cultivar that performs well in the field is not necessarily advisable for industry, as shown in **paper III**.

a) seedling of lettuce

b) commercially mature lettuce

Figure 2. (a) Seedling of lettuce and (b) commercially mature lettuce for harvest

Iceberg lettuce is very popular due to its crispy texture, attractive green leaves, neutral taste and green aroma (Rico et al., 2007). It is consumed fresh in salads, and it is a good dietary source of micronutrients such as vitamin A, C and E, and minerals such as calcium and iron (Hedges and Lister, 2005).

2.4. Respiratory metabolism of vegetables: an overview

While lettuce is growing it obtains all the energy it needs from the balance between utilization of carbon compounds (respiration) and acquisition (photosynthesis). However, once lettuce is harvested this balance is changed and the source of organic compounds is the built up reserves (Kays, 1991; Saltveit and Kader, 2003; Maguire et al., 2004). Respiration is the central process in living cells that release energy through the utilization of organic compounds, which is used to drive energy to catabolic and anabolic reactions inside the cell (Wills et al., 1982; Kays, 1991). Respiration can be aerobic (in the presence of oxygen) or anaerobic (in the absence of oxygen) (Wills et al., 1982). Under aerobic conditions, the complete oxidation of glucose involves three main reactions: glycolysis or Embeden-Meyerhof- Parnas (EMP) pathway, tricarboxylic acid cycle (TCA) or Krebs cycle and the electron transport system (Wills et al., 1982; Kays, 1999; Kader and Saltveit, 2003). A brief description of both types of respiration is given in the forthcoming subsections.

2.4.1. Glycolysis

Glycolysis occurs in the cytoplasm and produces two molecules of pyruvate from one molecule of glucose (Fig.3). The glycolysis involves a series of 10 enzymatic reactions, where the key enzyme of the process is the enzyme phosphofructokinase, which initiates the process (Kader and Saltveit, 2003). During the reaction two ATP (adenosine triphosphate) molecules and two NADH (reduced nicotinamide adenine dinucleotide) molecules are formed (Wills et al., 1982; Kays, 1999; Kader and Saltveit, 2003).

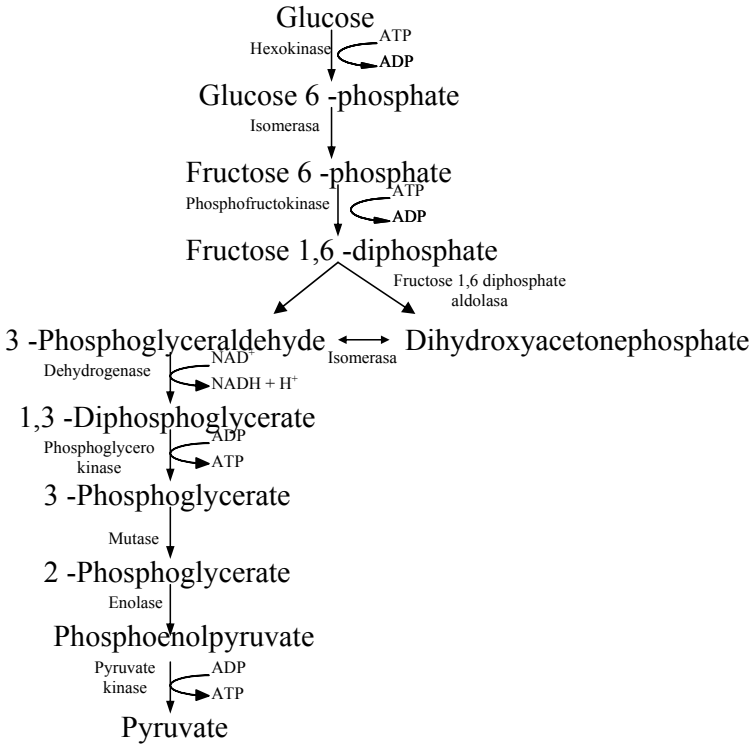


Figure 3. The glycolysis (Deza-Durand, 2006)

2.4.2. Tricarboxylic acid cycle (TCA)

The TCA cycle occurs in the mitochondrial matrix (Fig. 4). First, pyruvate moves by diffusion to the mitochondria, where it is descarboxylated to form acetate which condenses with a co enzyme A to form acetyl CoA. Further, acetyl CoA is condensed with oxaloacetate and enters to the cycle, in which, through seven sequential enzymatic reaction citric acid is formed (Wills et al., 1982). Citric acid is subsequently converted to oxaloacetate which readily reacts with another acetyl CoA molecule. Each molecule of pyruvate metabolized by TCA produces organic acids, three molecule of CO_2, one molecule of $FADH_2$ (reduced flavin adenine dinucleotide) and four molecules of NADH process (Kader and Saltveit, 2003).

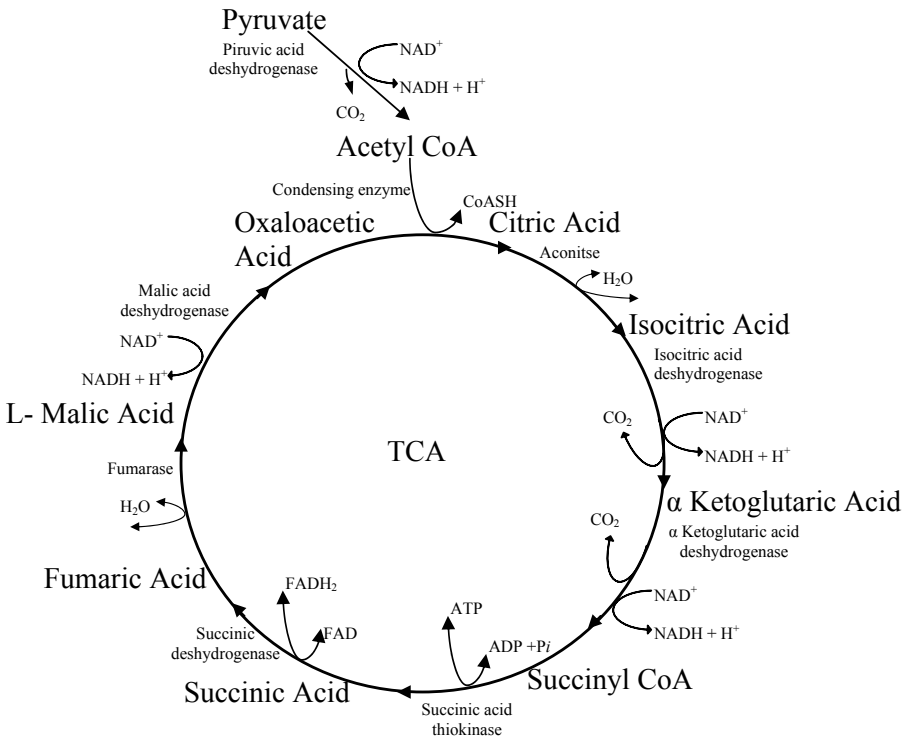


Figure 4. The tricarboxylic acid cycle (TCA) (Deza-Durand, 2006)

2.4.3. The electron transport system

The third pathway, the electron transport system occurs in the cristae of the mitochondria and involves the oxidation of $FADH_2$ and NADH obtained in the TCA and glycolysis to produce energy in form of ATP. In the process one NADH molecule produces three ATP molecules, while one $FADH_2$ molecule produces two ATP molecules (Wills et al., 1982; Kader and Saltveit, 2003).

2.4.4. Anaerobic respiration

In the absence of oxygen, glycolysis replaces the TCA cycle as the main source of energy for the plant tissue (Kader and Saltveit, 2003). The pyruvate is accumulated and further decarboxylated to acetaldehyde with a release of one molecule of CO_2. Subsequently, acetaldehyde is reduced to ethanol by the enzyme alcohol dehydrogenase with the

regeneration of NAD+. Two molecules of ATP are produced under anaerobic conditions against 37 produced under aerobic conditions (Wills et al., 1982; Kader and Saltveit, 2003).

2.5. Respiration rate of lettuce

Lettuce head has been classified as a commodity with moderate respiration rate (Kader, 2002). However, cutting lettuce for minimally processing speeds up the respiration rate (**paper II**, Kader and Saltveit, 2003). Respiration rate has been associated with the perishability of the product. Therefore it is assumed that higher respiration rate reduces the shelf-life of lettuce. Cutting direction has been indicated to affect the respiration rate of commodities such as tomatoes (Brecht, 1995) and green bananas (Abe et al., 1998). For lettuce it was demonstrated that the increase in respiration rate was higher in transverse than longitudinal cut sections, but temporary (**paper II**) as observed in Fig. 5 The increase of respiration rate in lettuce after cutting was due to enhanced aerobic mitochondrial respiration by enzymes such as phosphofructokinase and cytochrome oxidase (Asahi, 1978). However, this increase was observed until normal aerobic respiration is reestablished which depends on the commodity (Toivonen and DeEll, 2002). In addition, other methods of preparation such as shredding lettuce increases the respiration rate in comparison to cutting lettuce with a sharp knife or tearing by hand due to less damage to the tissue (Kader and Saltveit; 2003; Toivonen and DeEll, 2007)

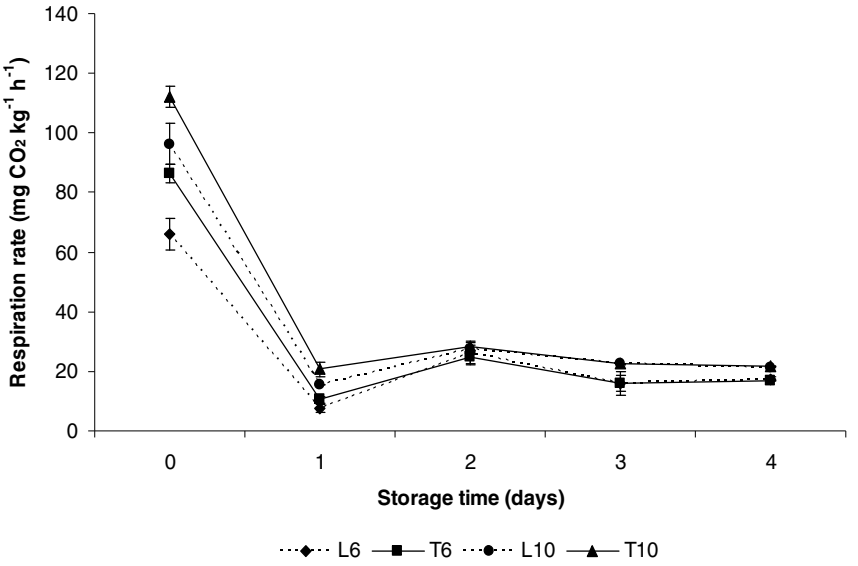


Figure.5. Respiration rate of iceberg lettuce cut transverse and longitudinal to the mid-rib stored in air at 6 and 10 ºC for 5 days in March 2008. *Abbreviations: L6, lettuce cut longitudinal stored at 6 ◦C; T6, lettuce cut transverse stored at 6 ◦C; L10, lettuce cut longitudinal stored at 10 ◦C; T10, lettuce cut transverse stored at 10 ◦C.*

In **paper II** and **IV** was found that respiration rate was mainly affected by storage temperature, season and maturity at harvest (Table 2 and 3) (Wills et al., 1982; Toivonen and DeEll, 2007). Analysis of variance showed significantly higher respiration rates of cut lettuce at 10 °C than at 6 °C of storage (**paper II**). Higher storage temperatures are expected to increase the respiration rate due to increased reaction rates of many pathways in cell respiration (Wills et al., 1982; Watada and Qi, 1999). In **paper II**, data from January exhibited significantly higher mean values of respiration rates than that from March, lettuce heads bought in January were mature, whereas those from March were over-mature, which could explain the trend to higher respiration rates in January due to young cells being more active than old cells (Wills et al., 1982; Kays, 1991).

Moreover in **paper IV**, mean value of respiration rate was around 100% higher in lettuces harvested in August in comparison with June, July and September ($p \leq 0.05$). Regarding the cultivars, respiration rate of cultivar Morinas and Platinas were

significantly higher than Diamantinas ($p \leq 0.05$), but these differences seemed to be minor in comparison with season as differences were mainly around 8%.

Table 2. Respiration rate of fresh-cut lettuce under different cutting directions stored at 6 and 10°C in January and March 2008.

Factors	Respiration rate ($mgCO_2\ kg^{-1}\ h^{-1}$)
Temperature of storage	
10°C	43.0± 30.8 (46) b
6°C	31.7± 22.4 (48) a
Replicates of the experiments	
January 2008	41.9± 21.1 (36) b
March 2008	34.3± 30.3 (58) a

Data expressed as mean ± standard deviation. Values on parenthesis represent the number of samples used for the calculation of the mean. Different letters indicate significantly differences at $p \leq 0.05$.

Table 3. Respiration rate of cultivars Platinas, Morinas and Diamantinas stored in air at 5 °C after 1 day of storage in June, July, August and September 2009.

Factors	Respiration rate ($mgCO_2\ kg^{-1}\ h^{-1}$)
Season	
June	59.3 ± 7.8 (9)[a]
July	60.2 ± 6.7 (9)[a]
August	112.5 ± 13.2 (9)[b]
September	60.3 ± 9.7 (9)[a]
Cultivars	
Platinas	76.7 ± 28.3 (12)[b]
Morinas	74.2 ± 28.1 (12)[b]
Diamantinas	68.3 ± 18.5 (12)[a]

Data expressed as mean ± standard deviation. Values on parenthesis represent the number of samples used for the calculation of the mean. Different letters indicate significantly differences at $p \leq 0.05$.

Anaerobic respiration is triggered when O_2 is depleted. This condition was observed in **paper IV and V**, passive MAP F1 and F2 allowed rapid development of anaerobic conditions after 5 days (0.02% O_2) with high accumulation of CO_2. Under this extreme atmosphere browning was not observed, but, odorants likely to be off-odors increased after 11 days of storage, loss of firmness and soluble sugars and malolactic fermentation was observed as well. Smyth et al (1998) also reported that at O_2 content between 0.3-0.5 % color retention is excellent and off-flavors were limited in packaged cut lettuce. The influence of extreme atmosphere on the formation of odorants and browning will be discussed in Chapter 4 and 5.

Chapter 3. Minimally processed lettuce

Minimally processed or fresh-cut vegetables are vegetables that have been cut in small pieces and are ready-to-eat (RTE) (Saltveit, 2003). Among them, iceberg lettuce is one of the most popular RTE vegetables. Preparing fresh-cut lettuce includes unit operations such as reception of raw material, coring, cutting, washing, centrifuging, packaging and storage (see Fig. 6). Processing of lettuce allows physical, biochemical and physiological changes to enhance the loss of quality in the product (Saltveit, 2003; Rico et al., 2007). Quality of a fresh-cut lettuce can be defined as "the combination of physical, chemical and sensory attributes of the produce, those are of importance to determine the degree of acceptability by the consumer" (Watada 1986; Rico et al., 2007).

The main limitation of fresh-cut lettuce's shelf-life is the development of browning (Heimdal et al., 1995, **paper I, V**), but off-odour is also a serious limiting factor (**paper III, IV**). The term "shelf-life" can be defined as the time period before the product attributes drop below the quality limit under specified storage conditions (Shewfelt, 1986; Rico et al., 2007). Browning and off-odour are still problems for the minimally processed industry in Denmark (GASA, oral communication). Therefore, this chapter will describe each step of minimally processing lettuce and its influence on browning and aroma of lettuce.

3.1. Raw material

The initial quality of a commodity to be minimally processed has high relevance in the final product (Shewfelt, 1986). In general, a high quality iceberg lettuce should be bright green, free of defects, crisp and turgid (Saltveit, 2000). Likewise, a compact head and adequate break-strenght of iceberg lettuce's leaves are characteristics highly desirable for the processor (GASA, oral communication). Maturity is also important for the processing of lettuce. Immature lettuce has soft and not fairly compact head which is difficult to cut by machine (Kader et al., 1973). On the contrary, overmature lettuce is ideal for cutting due to its hard and very compact state, but it is also more susceptible to postharvest

disorders during storage (Saltveit, 2000). Therefore, mature lettuce with a firm head is preferred because it provides a final product with better quality characteristics.

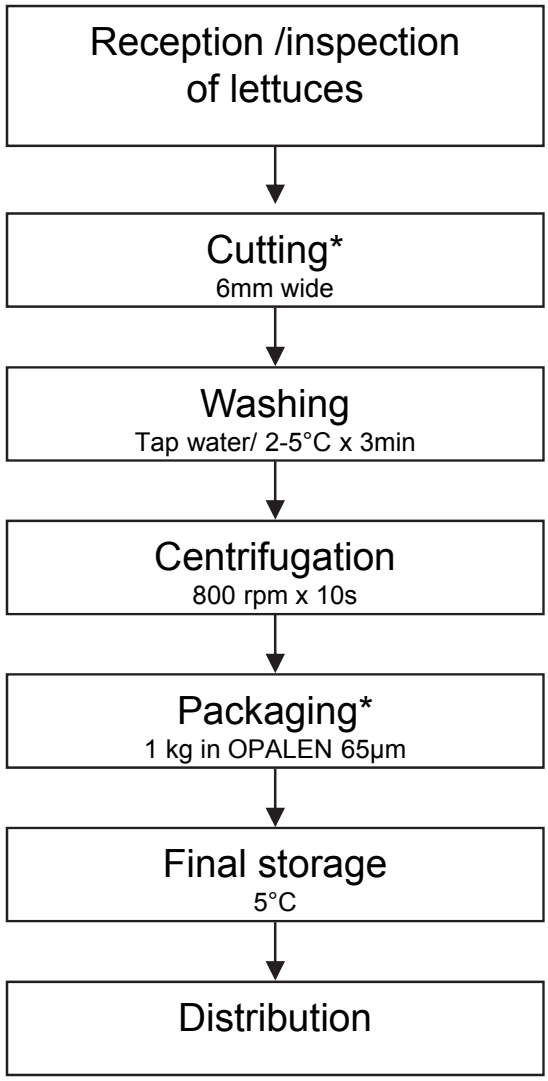


Figure 6. Minimally processed of iceberg lettuce

*: steps in the process examined in this thesis.

3.2. Cutting

The objective of cutting is to reduce the size of the vegetable, which improves product handling and provides a more convenient and time saving product (Sanguansri, 1997; Ragaert et al., 2004). At the factory external leaves and core of lettuce are removed manually before cutting (GASA, oral communication). Cutting of lettuce leads to the disruption of cells, promoting numerous physiological changes such as an increase in the respiration rate and enzymatic browning by mixing of polyphenol oxidase enzyme and phenolic acids, which reduce the shelf-life of the product (Couture et al., 1993; Saltveit, 2003). Likewise, tissue disruption releases enzymes which allow the formation of volatiles such as alcohols, aldehydes, terpenes, esters and acids (Belitz et al., 2004).

Methods of preparation of fresh-cut lettuce which minimize cutting damage are highly desirable. Bolin and Huxsoll (1991) found that tearing lettuce and cutting with a sharp knife led to a lower respiration rate and deterioration than shredding. Shredding is a term used to describe leafy vegetables cut in thin slices. Likewise, Martinez et al. (2008) found that a cutting grade less than 5 mm increase significantly the respiration rate of lettuce, which may lead to a higher speed of deterioration. Likewise, cutting direction influences the formation of volatiles in lettuce. In **paper II,** lettuce leaves were cut longitudinally and transversely to the mid rib. Transverse cutting was found to be a more severe method of preparation than longitudinal cutting, based on increased levels of volatiles produced through the LOX pathway.

3.3. Washing

The objective of washing is to remove exudates from cut tissue as well as soil and other possible contaminate, and to reduce the temperature of the produce (Kader and Saltveit, 2003). It is noteworthy that sanitizing agents such as chlorine are used in the wash water, mainly to reduce the microbial load in fresh-cut vegetables (Soliva-Fortuny and Martin-Belloso, 2003). Special attention has been appointed in the reduction of browning by the use of chlorine and warm water in the washing step (Baur et al., 2004; Delaquis et al., 2000). However the aroma of cut lettuce was affected negatively (Delaquis et al., 2000).

In Denmark the minimally processed vegetable industry doesn't use sanitizing agents (personal communication, Gasa) due to concern about the formation of harmful disinfection by the presence of products such as chloroamines and trihalometanes (Simons and Sanguansri, 1994). Instead, cold tap water is widely used to clean and sanitize the product and there has not been any indication of sanitation problems yet (personal communication, Gasa).

3.4. Centrifugation

The aim of centrifugation is to remove the excess of water retained by the product during washing (Moretti et al., 2007). Centrifugation is widely used in fresh-cut industry, but other methods such as vibration screen and forced air tunnel can be used for water removal (Bolin and Huxsoll, 1990; Moretti et al, 2007). For lettuce, it has been reported that slight desiccation of the produce improved the shelf-life of lettuce (Bolin et al., 1977; Bolin and Huxsoll 1991). However, an over-centrifugated product can increase the mechanical damage in the tissue i.e. cracks and crush, speeding up the loss of quality (Toole et al., 2000; Saltveit, 2003; Moretti et al., 2007).

3.5. Packaging

Modified atmosphere packaging (MAP) has been successfully used in cut lettuce to reduce browning by creating an atmosphere with low O_2 and high CO_2 (relative to air) and by storing at specific cold temperature (Zagory, 1998; Soliva-Fortuny and Martin-Belloso, 2003). Modified atmosphere can be created actively or passively. Active MAP is build up by flushing out air with a gas mixture (O_2, CO_2 and N_2) into the bag prior to sealing (Zagory, 2000). Passive MAP is developed by the interaction of packaging film gas permeability and respiration of the product (Talasila and Cameron, 1997). Passive MAP is used in the fresh-cut industry in Denmark. Since passive MAP depends on respiration rate of the commodity and film, it is important to choose a film that allows O_2 to enter at a rate that is consumed by the commodity and leave out the CO_2 at a rate that avoids extreme accumulation (Rakotonirainy et al., 2001; Saltveit et al., 2003). Low

density polyethylene, polyvinyl chloride and polypropylene are the main films used for packaging fruits and vegetables (Lee et al., 1996; Kader and Saltveit, 2003).

MAP has been successfully used for the reduction of enzymatic browning in cut lettuce (Heimdal et al., 1995; Smyth et al., 1998). However, extremely low O_2 and high CO_2 levels in the package can produce undesirable odours, alongside with a product with good appearance (Heimdal et al., 1995).

In **paper IV** and **paper V** lettuces were minimally processed and stored under three different treatments: two passive modified atmosphere packaging (MAP) built up by films of different permeabilities, F1 (OPALEN 65 AF) and F2 (OPP/PE-L 2040 AF), and storage in air. In table 4 is shown the different characteristics of film F1 and film F2. It was found that extremely low O_2 (<0.05%) and high CO_2 (>20%) built up by passive MAP F1 did not allow the formation of browning, however odorants likely to be off-doors increased after 11 days of storage. Moreover, the tolerance to extreme atmosphere for the formation of off-odours can change with the season and cultivar (Smyth et al., 1998). That was observed in **paper II** which is further discussed in Chapter 4.

Table 4. Characteristics of the films used in **paper IV** and **V**

Treatment	Film material	Transmission CO_2	Transmission O_2	Type of atmosphere
Passive MAP F1	OPALEN 65 AF (65 µm)	158	35	Passive MAP
Passive MAP F2	OPP/PE-L 2040 AF (60 µm)	Unknown	68	Passive MAP

The permeance data of the two films was provided by the manufacturer Bemis, Denmark.
Transmission rate CO_2 and O_2 (cm^3 m^{-2} 24 h^{-1} atm^{-1}), at 23 ºC and 50% RH for film F1 and at 23 ºC and 85% RH for film F2.
OPALEN: oriented polyamide/polyethylene.
OPP/PE-L: oriented polypropylene/linear low-density polyethylene.
MAP: Modified atmosphere packaging.

Chapter 4. Aroma of minimally processed lettuce

4.1. Volatile compounds and odorants identified in minimally processed lettuce

Most plant volatiles are lipophilic liquids with high vapor pressure (Pichersky et al., 2006). A total of 77 volatiles have been identified in this commodity (Table 5). Aldehydes, alcohols and terpenes constituted the main groups of volatiles. As observed in table 2, **paper II, III** and **IV** provide a major contribution of the number of volatiles identified in lettuce to our knowledge. Differences to other studies could be laid in differences of the analytical method as well as differences in the setup of the experiment, such as cultivar, growing conditions and packaging. Among the volatiles found, 20 were identified by GC-O as potent odorants in lettuce (Nielsen and Poll, 2006; Lonchamp et al., 2009; **paper IV**). Potent odorants are volatiles that contribute to the perceived aroma of food (Forney et al., 2000; Belitz et al., 2004). The contribution of a volatile to the aroma depends on its odor threshold and concentration in the food (Forney et al., 2000; Belitz et al., 2004). Cis-3-hexenal, cis-3-hexenol, trans-2-hexenal, 2-ethyl-1-hexanol, elemene, caryophyllene, copaene and (+) cyclosativene and 2-methoxy-3-isopropylpyrazine contributed to the green notes in lettuce (Nielsen and Poll, 2006; Lonchamp et al., 2009 **paper IV**). **Paper IV** showed that the number of odorants increased during storage time in packed cut lettuce. 2,3-Butanedione, 2-methylpropanal, hexanal, benzothiazole, β-selinene and five unknowns were detected only after 11 days of storage (Table 6). These compounds were most probably contributors to off-odour due to their sweet, rancid, unpleasant and spoiled vegetables aroma notes. Likewise, off-odour can arise from changes in the concentration of desirable compounds (Belitz et al., 2004; Poll et al., 2006). For example, in **paper II** was found that caryophyllene and/or elemene contributed to the leafy aroma of lettuce after 1 day of storage, but were after 11 days perceived as strong chemical due to higher concentration. The use of and/or indicates an uncertainty of which of both compounds was responsible for the odour due to problem

with identification between GC-MS and GC-O data. Off-odour can be described as an unpleasant odor that is not characteristic of lettuce aroma.

Table 5. Some volatiles compounds identified in iceberg lettuce

VOLATILE COMPOUNDS	*IDENTIFICATION*	*LITERATURE*
Aldehydes		
2-methylpropanal	GC-MS, GC-O	p(II); p(IV)
2/3-methylbutanal	GC-MS, GC-O	p(II); p(IV)
hexanal	GC-MS, GC-O	p(II); p(IV)
cis-3-hexenal	GC-MS, GC-O	a; b; p(II); p(IV)
trans-2-hexenal	GC-MS, GC-O	p(II); p(IV)
propanal	GC-MS	p(II); p(IV)
2-propenal	GC-MS	p(II); p(IV)
pentanal	GC-MS	p(II); p(IV)
Heptanal	GC-MS	p(II)
octanal	GC-MS	p(II); p(IV)
nonanal	GC-MS	p(II); p(IV)
2,4-hexadienal	GC-MS	p(II); p(IV)
decanal	GC-MS	p(II); p(IV)
acetaldehyde	GC-MS	e
benzaldehyde	GC-MS	p(II); p(IV)
Alcohols		
Ethanol	GC-MS	e
1-butanol	GC-MS	p(II); p(IV)
1-penten-3-ol	GC-MS	p(II); p(IV)
cis-3-hexenol	GC-MS, GC-O	a; b; p(II); p(IV)
1-propanol	GC-MS	p(II); p(IV)
2-methyl-1-propanol	GC-MS	c; p(II); p(IV)
2-methoxypropoxy-2-propanol	GC-MS	p(II)
1-pentanol	GC-MS	p(II); p(IV)
1-hexanol	GC-MS	p(II); p(IV)
trans-2-hexen-1-ol	GC-MS	p(II); p(IV)
2-ethyl-1-hexanol	GC-MS, GC-O	e; p(II), p(IV)
octanol	GC-MS	p(II); p(IV)
nonanol	GC-MS	p(II)
phenol	GC-MS	p(II); p(IV)
2-phenoxyethanol	GC-MS	e
3-mehtylbutanol	GC-MS	c
2-methylbutanol	GC-MS	c

3-methyl-1-pentanol	GC-MS	c
Terpenes		
caryophyllene and or elemene	GC-MS, GC-O	e, p(II); p(IV)
α-pinene	GC-MS	p(II); p(IV)
limonene	GC-MS	p(II); p(IV)
p-cymene	GC-MS	p(II); p(IV)
terpinolene	GC-MS, GC-O	d; p(II); p(IV)
α-humulene	GC-MS	p(II); p(IV)
α-selinene	GC-MS	p(II); p(IV)
β-selinene	GC-MS, GC-O	p(II); p(IV)
α-longipinene	GC-MS, GC-O	e; p(II)
α-muurolene		p(II)
copaene	GC-MS, GC-O	e
(+) cyclosativene	GC-MS, GC-O	e
(E)-α-bisabolene	GC-MS, GC-O	d
germacrene	GC-MS, GC-O	d
valencene	GC-MS, GC-O	d
Ketones		
2,3-butanedione	GC-MS, GC-O	p(IV)
3-hydroxy-2-butanone	GC-MS	p(IV)
2-butanone	GC-MS	p(II); p(IV)
6-methyl-5-hepten-2-one	GC-MS	p(II); p(IV)
acetophenone	GC-MS	p(II); p(IV)
Geranylacetone	GC-MS	p(II)
Acids		
acetic acid		
propanoic acid	GC-MS	p(II); p(IV)
butanoic acid	GC-MS	p(II); p(IV)
2-methyl butyric acid	GC-MS	p(II); p(IV)
hexanoic acid	GC-MS	p(II); p(IV)
Sulfur compounds		
dimethyl sulfide	GC-MS, GC-O	p(II); p(IV)
dimethyl sulfoxide	GC-MS	p(II); p(IV)
benzothiazole	GC-MS, GC-O	p(II); p(IV)
Esters		
ethyl formate	GC-MS	p(IV)
ethyl acetate	GC-MS	c; p(II); p(IV)
cis-3-hexenyl acetate	GC-MS	a; b; p(II)
propanoic acid ethyl ester	GC-MS	c
2-methylpropanoic acid ethyl ester	GC-MS	c
butanoic acid ethyl ester	GC-MS	c
3-methylbutanoic acid ethyl ester	GC-MS	c

3-methylbutylacetate	GC-MS	c
hexanoic acid ethyl ester	GC-MS	c
Pyrazine		
2-methoxy-3-isopropyl pyrazine	GC-MS, GC-O	p(II); p(IV)
Furans		
2-ethylfuran	GC-MS	p(II); p(IV)
2-pentylfuran	GC-MS	c; p(II); p(IV)
Miscellaneous		
ethyl tiglate	GC-MS	p(IV)
styrene	GC-MS	p(IV)

a: Arey et al., (1991); b: Charron et al., (1996); c: Smyth et al., (1998); d: Nielsen and Poll (2006); e: Lonchamp et al., (2009), p(IV): paper II, p(IV): paper IV.

Table 6. Some potent odorants found in packaged cut lettuce stored at 5 °C at 1 and 11 days of storage in September 2009 (**paper IV**).

Volatile compounds	Odor descriptor	
	1 day of storage	11 days of storage
2/3-methylbutanal	sweet, cacao	strong sweet, cacao
hexanal	n.d.	lettuce, fruity
trans-2-hexenal	vegetables	unpleasant, fatty
cis-3-hexenol	alcohol, chili, soil	soil, weak grass, tea
elemene and/or caryophyllene	lettuce, grass, flower, soil	strong chemical, grass, chili
dimethyl sulfide	boiled broccoli, shellfish	off-odour, broccoli, shellfish
2,3-butanedione	n.d.	sweet, caramel

n.d. = not detected

4.2. Plant biosynthetic pathways of volatile compounds

Volatiles in lettuce as well as in vegetables and fruits originate from fatty acids, amino acids and carbohydrate groups (Salunkhe and Do, 1976; Schwab et al., 2008). There is a lack of information on biosynthesis of volatiles in lettuce. It is assumed that volatiles in lettuce are formed through metabolic routes similar to those previously identified in tomatoes, carrots, broccoli, onion and other crops (Salunkhe and Do, 1976, Chin and Lindsay, 1993; Toivonen, 1997; Baldwin et al., 2000; Belitz et al., 2004; Reineccious, 2005). Differences in the volatile profile between lettuce and other vegetable crops, suggest that differences in the availability of substrate and or enzyme that determine the type and amount of formed volatiles exist, indicating that each vegetable specie is

capable of synthesizing their own characteristic volatile pattern (Salunkhe and Do, 1976, Pichersky et al., 2006). Likewise, in **paper II** and **IV** were found that mature lettuce produces a higher level of volatiles than over-mature lettuce. Moreover, differences in metabolic behavior between inner leaves (old leaves) and outer leaves (young leaves) and photosynthetic and vascular tissue could also influence in the formation of volatiles, as suggested in **paper II**. A better understanding of mechanisms of formation of volatiles in lettuce will provide a tool to control the aroma of lettuce.

The following section describes the possible routes for the formation of the main volatiles identified in this study.

4.2.1. Aldehydes and alcohols

Aldehydes can be formed from fatty acids that are oxidised via the lypoxygenase (LOX) pathway (Baldwin et al., 2000; Belitz et al., 2004). The pathway starts with the oxidation of linoleic or linolenic acid by LOX enzyme in presence of oxygen (Galliard et al., 1977). The mechanism of action is summarized in three steps: first, the activation of the native enzyme by the oxidation of Fe^{2+} to Fe^{3+}, subsequently, the removal of an H^+ from the substrate molecule complexed with the enzyme and finally the insertion of O_2 in the linoleic or linolenic acid at the position of carbon 9 or 13, resulting in 9-hydroperoxide or 13-hydroperoxide (Gardner, 1988; Robinson et al., 1995). As observed in Fig.7, the breakdown of hydroperoxides is further catalyzed by hydroperoxide lyase (HPL) to hexanal and cis-3-hexenal (Galliard et al., 1977; Baldwin et al., 2000). Likewise, cis-3-hexenal can be isomerized to trans-2-hexenal, either enzymatically or nonenzymatically (Galliard et al., 1977; Feussner and Wasternack, 1998; Baldwin et al., 2000). cis-3-Hexenal and trans-2-hexenal contributed to the green leafy aroma in lettuce (**paper IV**).

Aldehydes can also derive from branch-chain amino acids (Fig.8) (Belitz et al., 2004; Schwab et al., 2008). The reaction is initiated by aminotransferase forming 2-keto acid, which is further decarboxylated to produce branched chain aldehydes (Ardö, 1996; Marielly and Casey, 2004). Amino acids leucine, isoleucine and valine are converted to 3-methylbutanal, 2-methylbutanal and 2-methylpropanal, respectively (Ardö, 1996). The

sweet odor notes of these volatiles probably contributed to the off-odour in packaged cut lettuce after 11 days of storage (**paper IV**). Aldehydes can be reduced to their respective alcohols by the action of the enzyme alcohol dehydrogenase (Baldwin et al., 2000, Belitz et al., 2004). 1-Penten-3-ol and cis-3-hexenol were found to be potent odorants in fresh-cut lettuce (**paper IV**).

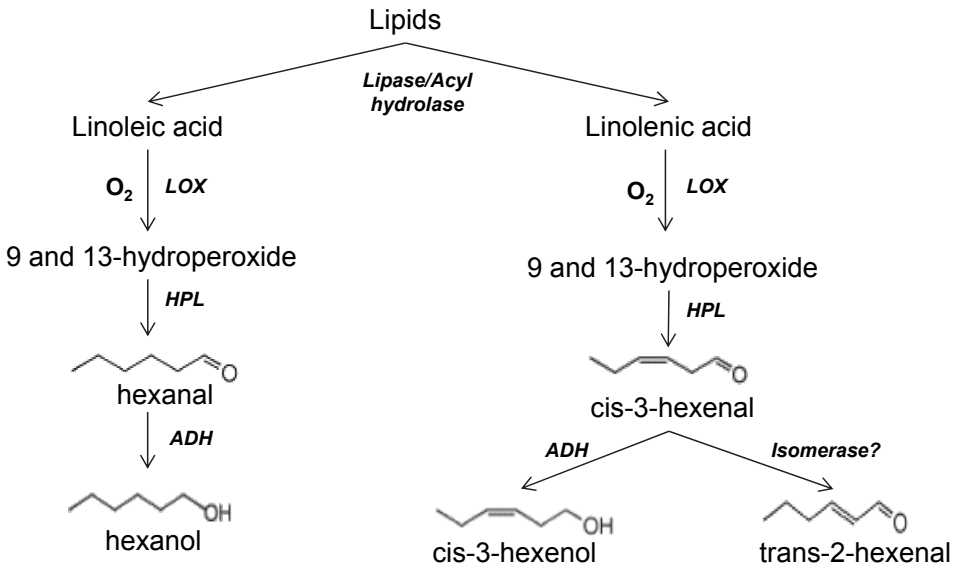


Figure 7. LOX pathway for the formation of C6 aldehydes and alcohols from degradation of lipids in plants (Gallaird et al., 1977; Gardner, 1995; Riley et al., 1996; Baldwin et al., 2000). LOX= lypoxygenase; HPL= hydroperoxide lyase; ADH= alcohol dehydrogenase.

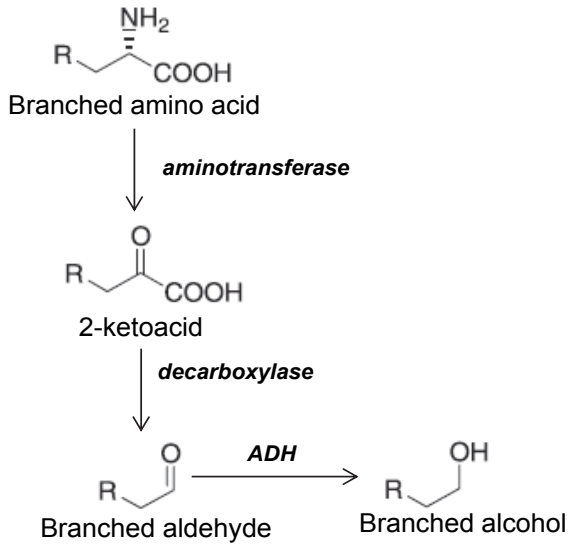


Figure 8. Pathway for the formation of branched aldehydes and alcohols from branched amino acids (Schwab et al., 2008). ADH= alcohol dehydrogenase.

4.2.2. Terpenes

Terpenes in the form of sesquiterpenes, such as caryophyllene, elemene and β-selinene contributed to the leafy aroma of cut lettuce (**paper IV**, Lonchamp et al., 2009). Sesquiterpenes are formed via the mevalonate (MVA) pathway in the cytosol (Pichersky et al., 2006; Tholl, 2006). The condensation of two molecules of isopentenyl pyrophosphate (IPP) and one molecule of its isomer dimethylallyl pyrophosphate (DMAPP) forms farnesyl pyrophosphate (FPP), which is subsequently catalyzed to sesquiterpenes by a terpene synthetase (Fig.9). In the plastids the methylerythritol phosphate (MPE) route also produces IPP and DMAPP molecules and is responsible for the formation of monoterpenes. Under certain conditions, such as stress, IPP and DMAPP molecules from this route can be transported from plastids to cytosol for the formation of sesquiterpenes (Piel et al., 1998; Hampel et al., 2004). It is believed that similar behavior might occur in packaged cut lettuce and cause the increase of caryophyllene and/or elemene under anaerobic conditions (**paper IV**).

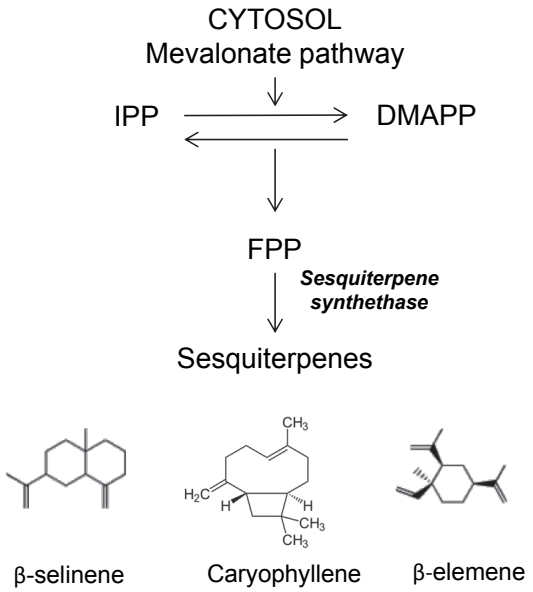


Figure 9. Pathway for the formation of terpenes in plants (Pichersky et al., 2006; Tholl, 2006)

4.2.3. Volatiles from anaerobic metabolism

MAP with extremely low O_2 (<1%) and high CO_2 (>20%) may lead to anaerobic respiration with the formation of off-odours (Kader, 1997). In our study 2,3-butanedione, 3-hydroxy-2-butanone, ethyl acetate and ethyl formate were identified as volatiles from anaerobic metabolism (**paper III** and **IV**). Among them only 2,3-butanedione was identified by GC-O (**paper IV**), which likely contributes to the off-odour in cut lettuce due to its low odor threshold (5ppb) (Burdock, 2005). Previous work by Smyth et al. (1998) identified ethanol, acetaldehyde and short-chain methyl-branched alcohols as volatiles from extremely low O_2 in packaged cut lettuce.

The formation of these volatiles under anaerobic conditions is the result of accumulation of pyruvate by the Emden-Meyerhof pathway in the cytoplasm. Pyruvate is then converted to acetaldehyde by pyruvate descarboxylase and further to ethanol by the action of alcohol dehydrogenase. The high level of CO_2 reduces the pH of the cytoplasm, which enhances the activity of the enzymes previous mentioned (Siriphanich and Kader, 1986; Ke et al., 1994). As a result an increasing level of ethanol may also stimulate the

formation of ethyl esters such as ethyl acetate and formate (Larsen, 1994; Forney et al., 2000).

4.3. Analytical methods for volatile analysis in lettuce

In **paper II**, **III** and **IV** volatiles were isolated from homogenized lettuce using dynamic headspace sampling. For separation and identification of volatiles in cut lettuce a gas chromatography (GC) coupled with a mass spectrometry (MS) was used. However, this technique does not provide information of the contribution of an individual compound to the aroma of lettuce. To this end, gas chromatography olfactometry (GC-O) was used. Table 7 shows the principle and the aim behind the analysis of volatiles. Further description of the operation parameters of the GC-MS and GC-O (FID) equipment can be found in **paper II** and **IV**.

Table 7. Principles of the analytical techniques for volatile analysis in lettuce (**paper II**, **IV**).

Technique	Aim	Principle
Dynamic headspace sampling	Isolation of volatiles (purge and trap)	Sample volatiles were constantly purged from an aqueous matrix to a trap (Tenax), which allowed the enrichment of more volatiles from the sample, increasing sensitivity of the analysis. Volatiles trapped in an adsorbent were thermally desorbed direct to the GC.
Gas chromatography-mass spectrometry (GC-MS)	GC Separation	Volatiles were swept by a carrier gas (mobile phase) through the column. Stronger interaction between the volatile and the column inner surface (stationary phase) leaded to longer retention time. This interaction is temperature dependent. For an adequate separation of the volatiles the GC was operated in a temperature range between 40 and 240 ºC.
	MS Identification	Each volatile eluted direct to the detector. There, the volatile was ionized and fragmented and the ions formed were separated according to their mass/charge ratio. The output from the analysis was a mass spectrum which was used for identification.

Gas chromatography-Olfactometry (GC-O)	To determine the potent odorants in a sample	GC-O was performed in a conventional GC equipped with a sniffing port. The flow from the column was split between the detector (flame ionizant detector) and the sniffing port, where the human nose was used as a detector, providing simultaneous detection of the potent odorants. From each individual volatile that elute from the port, the judge was instructed to describe the odour and retention time.

In GC-O analysis, the human nose is used as a detector (Pollien et al., 1997). It is known that the olfactory system is more sensitive than any physical detector e.g. FID and MS to some compounds (Delahunty et al., 2006).

For the identification of the potent odorants in **paper IV**, the samples were run in duplicates, with the GC-O and GC-MS under the same conditions. Likewise, a standard mixture of references was run in the GC-MS and GC-O equipment. The GC-O retention time of the sample and GC-O and GC-MS retention time of the reference mixture was interpolated to found the potent odorant in the GC-MS chromatogram of the sample. Once the potent odorants were identified in the GC-MS chromatogram, their retention time was plotted against the retention time of the GC-O for the same sample, see Fig.10. As can be seen in Figure 10 all points fall within the tendency, soft s-shape, which confirms that the identification of potent odorants was as expected. The use of and/or in elemene and/or caryophyllene indicates an uncertainty of which of both compounds was responsible for the odour.

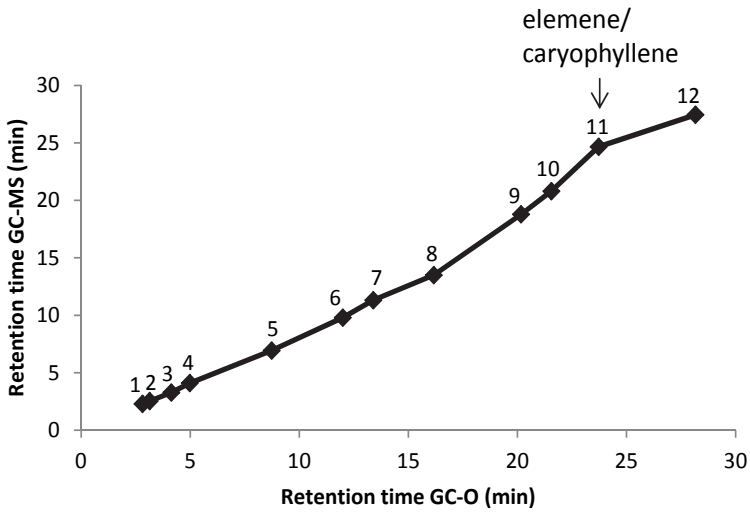


Figure 10. Plot of GC-MS retention time vs. GC-O retention time for the identification of potent odorants. *Abbreviations: 1: dimethyl sulfide, 2: 2-methylpropanal, 3: 2/3-methylbutanal, 4: 2,3-butanedione, 5: hexanal, 6: cis-3-hexenal, 7: 1-penten-3-ol, 8: trans-2-hexenal, 9: cis-3-hexenol, 10: 2-methoxy-3-isopropylpyrazine, 11: elemene/caryophyllene, 12: β-selinene.*

Therefore, identification of the potent odorants was also examined by using retention index (RI). Figure 11 shows the plot of retention index against the GC-MS retention time of some identified potent odorants. Retention index was taken from literature, (Sumitami et al., 1994; Cha and Cadwallader, 1998; Le Guen et al., 2000; Ruther, 2000; Pennarum et al., 2003; Varming et al., 2004; Gancel et al., 2005; Chen et al., 2009; Mesa-Arango, 2009) where DBWAX column was used. From Figure 11, it can be seen that elemene and caryophyllne elute too close as such correctly identified. Elemene and caryophyllene are terpenes with similar aroma descriptions in literature (www.flavournet.org) that correspond to the description provided by the judges. Therefore, due to uncertainty to discriminate which of them produce the odor it was decided to use and/or for these compounds.

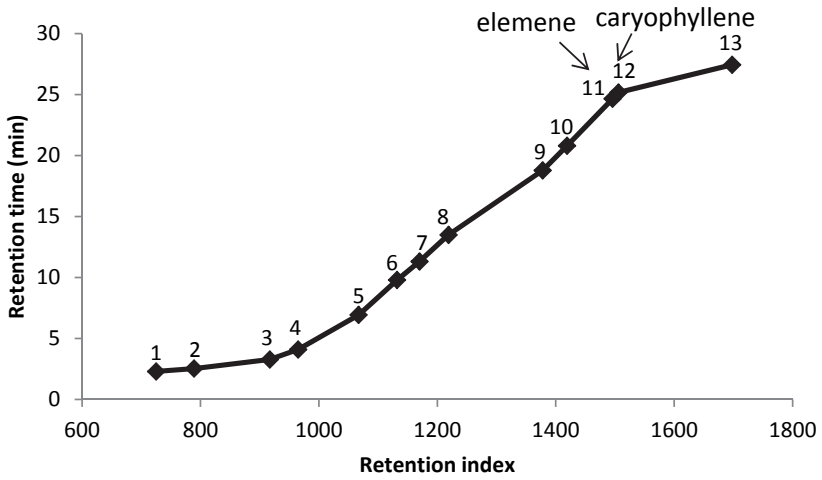


Figure 11. Plot of GC-MS retention time and RI of potent odorants. *Abbreviations: 1: dimethyl sulfide, 2: 2-methylpropanal, 3: 2/3-methylbutanal, 4: 2,3-butanedione, 5: hexanal, 6: cis-3-hexenal, 7: 1-penten-3-ol, 8: trans-2-hexenal, 9: cis-3-hexenol, 10: 2-methoxy-3-isopropylpyrazine, 11: elemene, 12:caryophyllene, 13: β-selinene.*

4.4. Effect of important factors in the formation of volatiles in cut lettuce

Cultivar, season, method of preparation, packaging, temperature and storage time influence the type and concentration of volatiles in vegetables (Smyth et al., 1998; Hodges and Toivonen, 2008, **paper II, III, IV**).

Few studies are done on the formation of volatiles in fresh-cut lettuce. Among them, the investigations were focused on the effect of packaging, storage temperature and time (Smyth et al., 1998; Lonchamp 2009). In **paper IV** a more integrated study was undertaken for first time in lettuce to our knowledge. The study took into account the influence of cultivar, season as well as packaging, storage temperature and time. Likewise, the potential of producing volatile compounds due to the direction of the cut (method of preparation) in combination with storage temperature was discussed for first time in **paper II**.

In this chapter, **paper II, III** and **IV** will be outlined for the discussion regarding factors that affect the volatile compounds in lettuce.

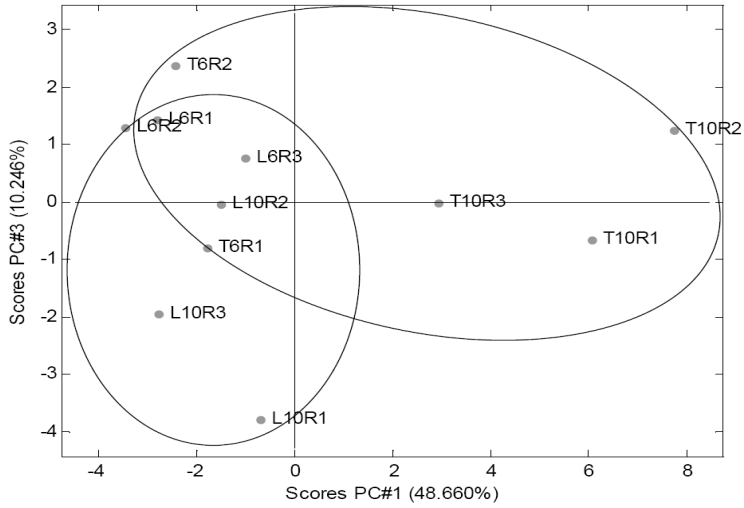
4.4.1. The effect of the method of preparation and temperature of storage

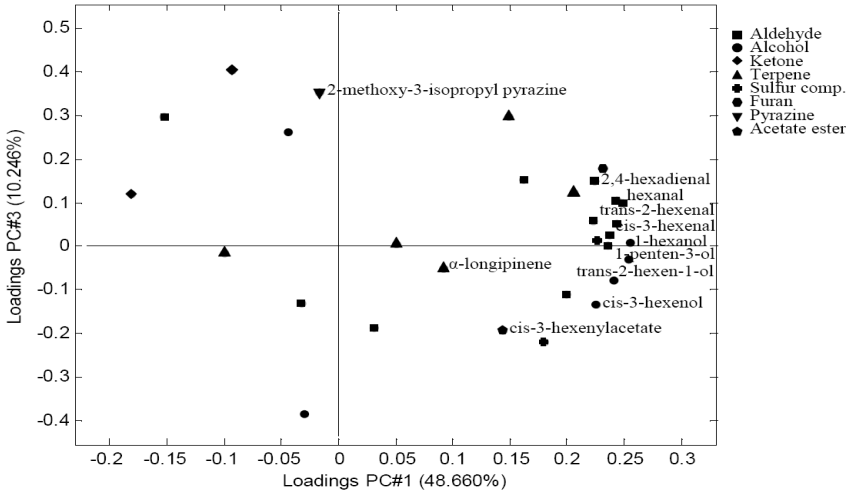
Paper II*: The effect of cutting direction on the aroma compounds of fresh-cut iceberg lettuce*

The cutting direction in combination with higher storage temperatures appears to play a significant role in the formation of volatiles in lettuce. In **paper II** iceberg lettuce was cut longitudinal and transverse to the mid-rib and stored in air at 6 and 10ºC for 4 days in January 2008 and 5 days in March 2008. Volatiles were isolated at the end of the storage using dynamic headspace sampling and identified by GC-MS.

A PCA plot (Fig. 12a) of data from January 2008 shows that volatiles from the LOX pathway were higher in the transverse cut samples stored at high temperature. Particularly, 1-penten-3-ol, hexanal, hexanol, 2,4-hexadienal and trans-2-hexenal, were found to be significantly higher when lettuce was cut transversely and stored at 10ºC ($p \leq 0,05$). Among the compounds, trans-2-hexenal was the most affected. It increased up to 10 times more than other LOX volatiles. Furthermore, in the March 2008 data (Fig. 12b), cis-3-hexenal and cis-3-hexenol were also strongly associated with transverse cutting but at 6 °C, while trans-2-hexenal, trans-2-hexen-1-ol, 2-ethyl-1-hexanol, 2,4-hexadienal, hexanal, 1-hexenol and 1-penten-3-ol seemed to be related to higher storage temperatures. LOX has been shown to be a stress-related enzyme (Hildebrand, 1989), and an increase in these compounds by cutting the lettuce in the transverse direction might indicate a greater disruption of membranes, which in combination with high storage temperature probably increased activity of enzymes related to senescence and lipid degradation such as acyl hydrolase and LOX.

a) January 2008





b) March 2008

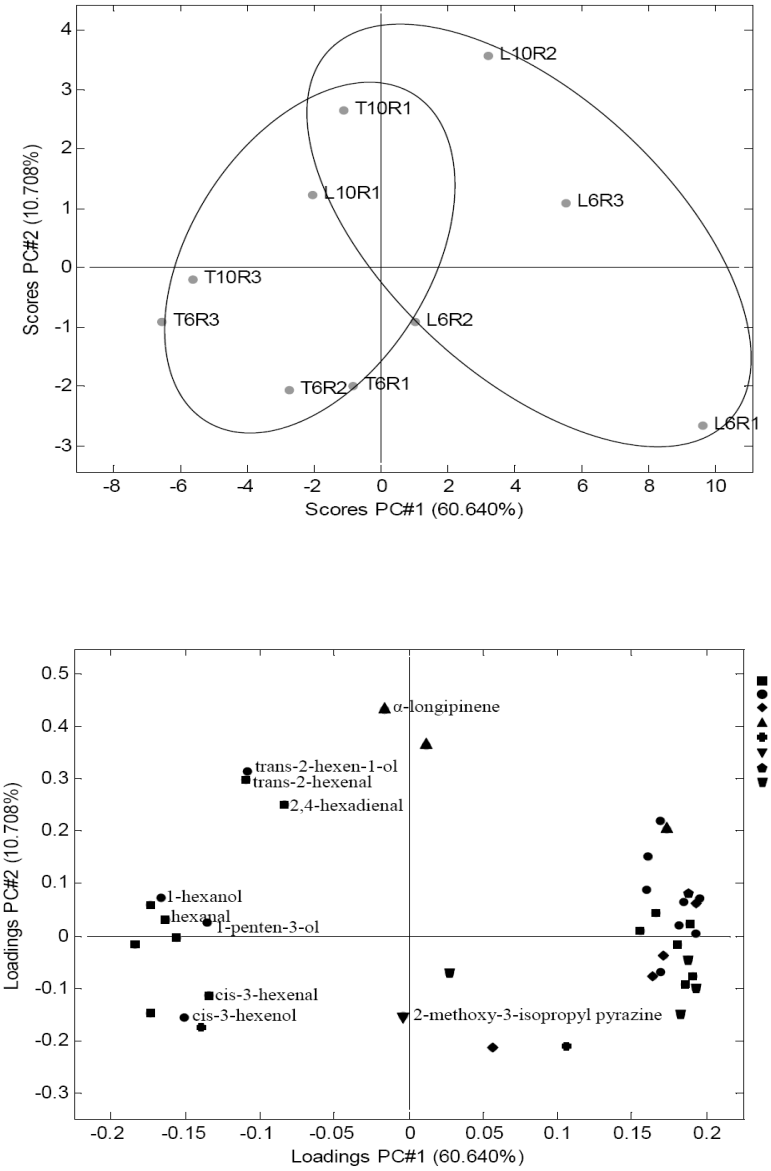


Figure 12. PCA analysis of fresh-cut lettuce cut transverse and longitudinal stored in air at 6 and 10°C after 4 days in January 2008 (a), and after 5 days in March 2008 (b). *Abbreviations: T6= Lettuce cut transverse stored at 6°C, L6= Lettuce cut longitudinal stored at 6°C, T10= Lettuce cut transverse stored at 10°C, L10= Lettuce cut longitudinal stored at 10°C, Replicates= R1, R2, R3.*

The state of maturity of lettuce also had an effect on the production of LOX volatiles between experiments. For example, in January, lettuce heads were mature, which means that LOX activity might be high in comparison with overmature lettuce found in March. Matsui et al. (1997) showed that LOX activity is reduced with an increase in maturity. Moreover, the diversity of volatiles from other enzymatic reactions observed in the March samples from the longitudinal cutting (Table 8) could also be influenced by the stage of maturity. Further research is needed on the metabolic routes for volatile formation in lettuce and their relation to severity of tissue damage and/or to different parts of lettuce that could have different metabolic behavior i.e. inner and outer leaves, photosynthetic and vascular tissue.

Table 8. Interaction between experiment and type of cutting on the relative area of aroma compounds of lettuce stored in air for 4 and 5 days in January and March.

Experiment	January 2008		March 2008	
Cutting	Transverse	Longitudinal	Transverse	Longitudinal
Aroma compounds				
2-butanone	0^{a}	0^{a}	0.03^{a}	0.06^{b}
1-butanol	0^{a}	0^{a}	0.01^{a}	0.03^{b}
1-pentanol	0^{a}	0^{a}	0.002^{a}	0.01^{b}
1,2-methoxypropoxy -2-propanol	0^{a}	0^{a}	0.02^{a}	0.05^{b}
octanol	0^{a}	0^{a}	0.0026^{a}	0.01^{b}
phenol	0^{a}	0^{a}	0.02^{a}	0.07^{b}
propanoic acid	0^{a}	0^{a}	0.0035^{b}	0.01^{c}
butanoic acid	0^{a}	0^{a}	0.01^{a}	0.03^{b}
ethyl acetate	0^{a}	0^{a}	0.01^{a}	0.02^{b}
nonanal	0.01^{ab}	0.01^{a}	0.03^{b}	0.05^{c}
decanal	0.01^{a}	0.01^{a}	0.01^{b}	0.03^{c}
benzaldehyde	0.01^{a}	0.01^{a}	0.02^{a}	0.03^{b}
limonene	0.02^{ab}	0.01^{a}	0.02^{a}	0.05^{b}

Values with different letters across a row are significantly different ($p \leq 0.05$).

4.4.2. The effect of season, cultivar, packaging and storage time

***Paper IV**: Effect of season, cultivar, packaging and storage time on volatile formation of iceberg cut lettuce*

In **paper IV** changes in volatile compounds of minimally processed iceberg lettuce was measured as a function of season, cultivar, packaging and storage time. In order to achieve this, iceberg lettuce cultivars Platinas, Diamantinas and Morinas were harvested from June to September 2009. Lettuces were minimally processed and stored under three different treatments: two passive modified atmosphere packaging (MAP) built up by films of different permeabilities, F1 (OPALEN 65 AF) and F2 (OPP/PE-L 2040 AF), and storage in air. All packages were stored at 5°C. Volatile compounds were assessed at 1, 5, 8 and 11 days of storage in packaged lettuce, whereas in air stored samples volatiles were analyzed only at 1 and 5 days of storage. Additionally, GC-O analysis was undertaken after 1 and 11 days of storage for cultivar Morinas packaged in passive MAP F2 in September.

Fifty two volatiles were identified in this study; of these 21 including 8 unknowns were identified by GC-O as potent odorants. A PLS-DA was done to discriminate the odorants within season. To this end a model was developed using the 13 potent odorants identified by GC-O and gas composition of passive MAP and air stored samples of cultivars Platinas, Morinas and Diamantinas stored for up to 11 days in June, July, August and September 2009 (Fig. 13). From the score plot can be seen that PC1 and PC2 discriminate within season. Changes in potent odorants within season could be attributed to climatic conditions, which could explain their position along PC1. In the other hand, lettuces harvested in June and August were mature in comparison with over-mature lettuces harvested in July and September, which might have attributed their position along PC2. In order to understand the formation of odorants within season, a comparison between months with lettuces with same maturity was undertaken, as shown in the loading plot of Fig. 13. For examples, in June cooler temperatures and long days may have promoted the synthesis of amino acid such as valine and s-methylmethionine for the formation of 2-methoxy-3-isopropyl pyrazine and DMS. In contrast, August was mainly

characterized by having high air temperature. This condition stressed the lettuce by the fact of a high metabolism was found in August. This could reduce the tolerance of cut lettuce to anaerobic conditions and accelerate the deterioration, resulted in an enhancement of 2, 3 butanedione, caryophyllene, β-selinene and elemene, which are likely to be off-odours. This indicates that probably it would be more difficult to maintain low production of potent odorants likely to be off-odours in August.

Figure 14 shows the score and loading plot of a PLSDA for discrimination between storage time. From the score plot, it can be seen that all the samples were displaced clockwise from day 1 to day 11 of storage. The displacement of samples evidenced changes in the amount of potent odorants as storage time increased. After 1 day of storage, aerobic conditions predominated in the passive MAPs and air stored samples, which seemed to increase the level of cis-3-hexenol. As storage time increased the level of O_2 decreased and the CO_2 content increased in the passive MAPs. Therefore, most of the odorants seemed to increase after 8 days of storage, after being exposed to extremely low O_2 and high CO_2. Further exposition, up to 11 days, enhanced the formation of odorants that were described as unpleaseant such as 2,3-butanedione, β-selinene, caryophyllene and elemene. Changes in the formation of odorants as storage time increased could be the result of membrane deterioration by prolonged exposition to extremely low O_2 and high content, mainly developed in passive MAP F1 which enhanced the formation of odorants likely to be off-odours (Chin and Lindsay, 1993).

In general, the results indicated that mainly season and storage time and in less degree cultivar influence the formation of potent odorants packaged and air stored cut lettuce. Among cultivars, differences in the formation of individual potent odorants were not significant, with exception of 2,3-butanedione which was significantly higher in the cultivars Morinas and Diamantinas after 11 days of storage in passive MAP F1 in August ($p \leq 0.05$).

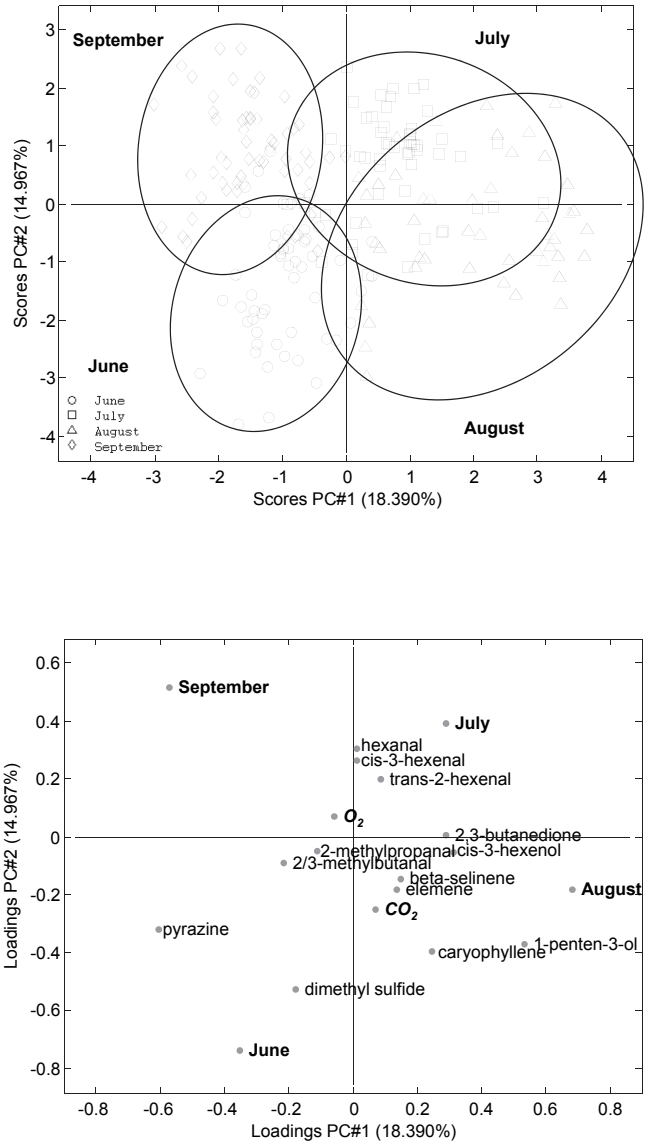


Figure 13. A PLS-DA score and loading plots of potent odorants and gas composition from passive MAP and air stored samples of cultivars Platinas, Morinas and Diamantinas stored for up to 11 days at 5 ºC in June, July, August and September 2009.

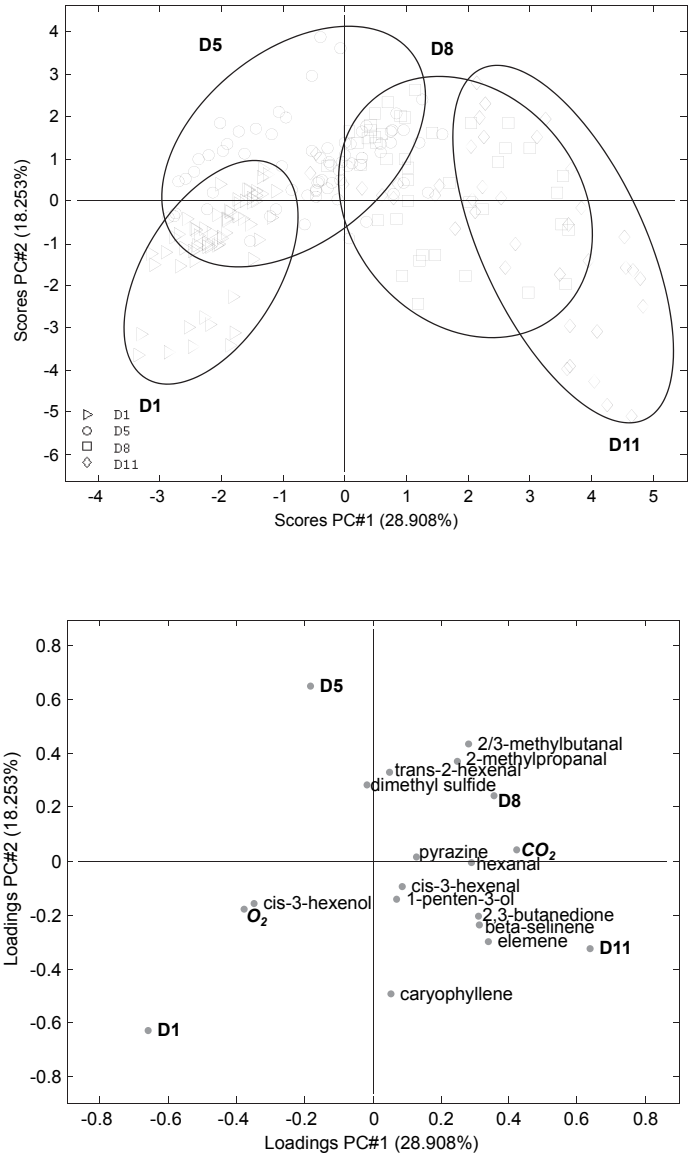


Figure 14. PLS-DA score and loading plots of odorants and gas composition of data from passive MAPs and air stored samples of cultivars Platinas, Morinas and Diamantinas harvested in June, July, August and September 2009 stored for up to 11 days at 5°C. *Abbreviations: D1, first day of storage; D5, fifth day of storage; D8, eighth day of storage; D11, eleventh day of storage*.

Chapter 5. Enzymatic browning and other physicochemical characteristics of minimally processed lettuce

5.1. Formation of browning in minimally processed lettuce

Browning has been reported as the main limitation of the shelf-life of minimally processed lettuce (Heimdal et al., 1995). The mechanism of enzymatic browning in lettuce is initiated by cell damage by cutting, which allows the interaction of polyphenol oxidase (PPO) and phenolic compounds (Toivonen and Brummell, 2008). As a result, quinones are formed which react non-enzymatically with other quinones, amino acids or proteins to produce melanin pigments, responsible for the brown color in the cutting edge of lettuce (Ramirez et al., 2003; Doğan and Salman, 2007). It is noteworthy that there are other enzymes involved in this process, such as phenylalanine ammonia-lyase, which leads with the biosynthesis of phenolic acids, and peroxidase, that can also form melanines, but its role depends on the presence of H_2O_2 in the cell, which is generally very low (Lopez-Galvez, 1996; Richard-Forget and Gauillard, 1997; Fujita et al., 2006). PPO has been indicated as the key enzyme for the development of enzymatic browning (Martinez and Whitaker, 1995). In this study, special attention has been on polyphenol oxidase due to its influence in enzymatic browning.

5.2. The PPO: An overview

PPO has been isolated from different sources such as bacteria, fungi, arthropods, mammals and plants. In plants PPO has been found in the plastids in soluble form and membrane-bound (Martinez and Whitaker, 1995).

PPO is an oxidoreductase that catalyses the oxidation of phenolic acids in presence of O_2 (Ramirez et al., 2003). PPO is able to catalyze two different reactions: 1) hydroxylation of monohydroxyphenols and 2) the oxidation of *o*-dihydroxyphenols to *o*-quinone (Ramirez et al., 2003; Doğan et al., 2007). In lettuce, PPO from vascular and photosynthetic tissue is specific in their cleavage for *o*-dihydroxyphenol and can be

classified as a catechol oxidase (E.C.1.10.3.1) (Heimdal et al., 1994; Ramirez et al., 2003; Doğan and Salman, 2007).

The *o*-dihydroxyphenol oxidase activity involves the oxidation of 2 molecules of substrate to obtain 2 molecules of *o*-quinone (Ramirez et al., 2003). The proposed mechanism of oxidation of *o*-dihydroxyphenols is shown in Fig. 15. The active site of PPO has two copper atoms that show different functional states during the catalytic activity*: met, deoxy* and *oxy* (Ramirez et al., 2003). The mechanism of action can be summarized in 5 steps: 1) the addition of a substrate (*o*-dihydroxyphenol) binds to the *met* form [Cu(II)], 2) producing the *deoxy* form of the enzyme [Cu (I)] and a molecule of *o*-benzoquinone, subsequently, 3) the *deoxy* form bind the O_2 to give the *oxy* form [Cu(II)], 4) which then bind a molecule of catechol to give the ternary complex enzyme Cu(II).O_2. *o*-dihydroxyphenol, 5) finally two hydrogens are removed to obtain the *o*-quinone and the *met* form of the enzyme completing the cycle (Lerch, 1983; Solomon et al., 1992; Ramirez et al., 2003).

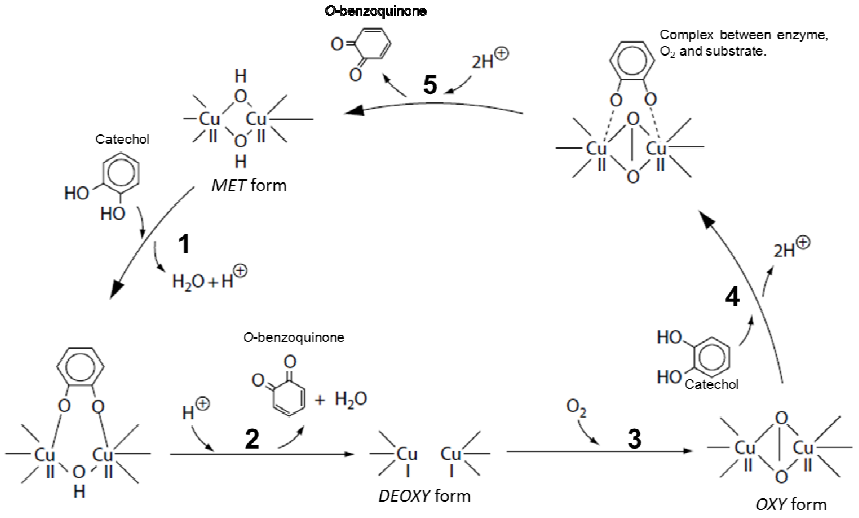


Figure 15. Proposed mechanism of action of PPO for oxidation of *o*-dihydroxyphenol using catechol as substrate (Lerch, 1983; Solomon et al., 1992; Ramirez et al., 2003).

The *o*-quinones are very reactive non-enzymatically with O_2, other quinones, sulfhydryl compounds, amines, amino acids and proteins to produce melanin pigments responsible for the brown color (Ramirez et al., 2003; Doğan and Salman, 2007).

5.3. The PPO substrates in lettuce

In lettuce potential *o*-dihydroxy substrate for PPO are chlorogenic acid (5-*O*-caffeoylquinic acid), chicoric acid (dicaffeoyl tartaric acid), isochlorogenic (dicaffeoylquinic acid) acid and caftaric acid (caffeoyl tartaric acid (Cantos et al., 2001; Baur et al., 2004; Sobolev et al., 2005). The main storage organelle of these compounds is the vacuole (Queiroz et al., 2008), but chlorogenic acid and chicoric acid have also been found located in chloroplast, epidermis and vascular boundles in lettuce (Heimdal, 1995). Once lettuce is cut, chlorogenic acid is accumulated in lettuce midrib caused by a *de novo* formation by enzyme phenylalanine amonnia lyase (PAL) (Ke and Saltveit, 1989; Cantos et al., 2001). For instance, in **paper V** was found a high content of chlorogenic acid with 87.21 mg/100g of fresh weight (FW) in cut lettuce stored in air after 5 days of storage at 5ºC, whereas in packaged cut lettuce, passive MAP F2 and F1, the level was 48.98 and 35.99 mg/100g FW, respectively, probably due to differences in *de novo* biosynthesis of phenolic acids by PAL enzyme under high CO_2 (Mateos et al., 1993).

5.4. Analysis of enzymatic browning in lettuce

5.4.1. Polyphenol oxidase activity

In **paper V** a spectrophotometric method was used for the measurement of PPO activity in cut lettuce. Spectrometry methods are based on changes in absorbance of the product or substrate as a function of time (Copeland, 1996). Polarographic methods have also been used for PPO activity in lettuce (Heimdal et al., 1995). The latter method measures the oxygen depletion during the enzyme reaction (Copeland, 1996).

Irrespective of the method, the assay of bisubstrate reactions is performed as for pseudo-monosubstrate reaction (Sørensen et al., 1999). Initial velocity of PPO can be determined by maintaining one substrate, e.g. O_2, at high concentration relative to the other substrate (e.g. chlorogenic acid), where O_2 concentration can be considered constant during the reaction (Sørensen et al., 1999). During the assay, PPO is irreversible inactivated. Inactivation is due to a free radical-catalyzed fragmentation of one or more of the six histidine residues of the enzyme that bind the two coppers at the active site (Ramirez et al., 2003).

Some characteristics of PPO of iceberg lettuce have been described by Heimdal et al. (1994) and Gawlik-Dziki et al. (2008). Heimdal et al. (1994) reported for both vascular and photosynthetic tissue, a pH optimum ranged from 5 to 8 with an optimum temperature between 25 to 35 ºC, using chlorogenic acid as substrate.

5.4.2. Image analysis

Objective measurements of browning in cut lettuce have been made using colorimeters (Heimdal et al., 1995). Measurements with colorimeters usually takes several points to compensate the un-uniformly surfaces of cut lettuce and then provide an average colour, which might not represent the colour of the sample (O'Sullivan et al., 2003). Therefore, to overcome this problem, an image analysis of cut lettuce was developed in **paper I** and used for evaluations of browning in **paper V**.

For image acquisition a flatbed scanner was used, which provided a better representation of colour by the fact that the flatbed scanner captures a bigger area of the sample, around 600 times of a Minolta colorimeter. When images are captured changes in lighting conditions of the scanners could affect the colours in the image obtaining unreliable information for comparison. In order to avoid this problem, a colour correction was performed.

For colour correction a transformation was sought to each image to bring the colours of the reference patches to match a reference image. The mean RGB pixel values of each

colour patch in the image to be corrected were used to create a 20x3 matrix P_I of pixel values with one row per patch. A least square linear transformation into the corresponding matrix P_R for the reference image was obtained as follows:

$$L= [P_R^T P_R]^{-1} P_R^T P_I$$

To obtain the corrected pixel value p_c for each pixel in the image we computed:

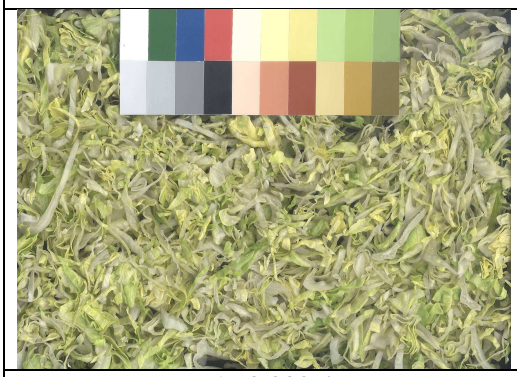
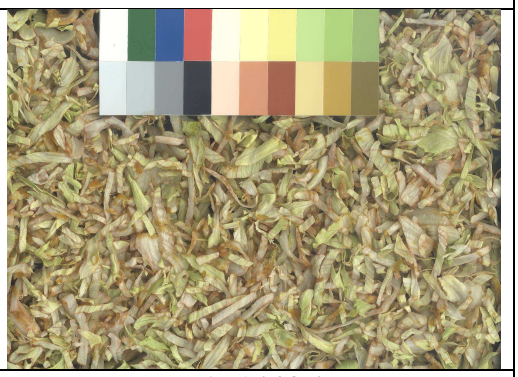
$$p_c=L*p$$

where p is the uncorrected RGB pixel value.

Once images were colour corrected they were used for further analysis. A thresholding to extract pixels of brown colour was applied to each corrected image and the brown area fraction (BA) was calculated as explained in **paper I**. Thresholding is a selection of a range of brightness, hue and saturation that allowed the extraction and quantification of the browning in the images, which was perceived as a pinking colour adjacent to the wound area.

In **paper I** it was demonstrated that image analysis is a useful technique for measuring browning in cut lettuce. This method provides a better representation of colour by using a big area of sampling and accurate quantification of browning by thresholding the colour corrected images (Fig. 16).

Figure 16. Brown area fraction (BA) of cut lettuce at different storage conditions.

0 days at 5 °C	Day 7 (6 days at 5°C plus 1 day at room temperature)
	
BA=0.003%	BA=15.23%

5.5. Important physicochemical characteristic of minimally processed lettuce: texture, soluble sugars and organic acids

5.5.1. Firmness

Texture is defined as the sensory expression (touch, vision, and hearing) of the structural elements of a food and how this structure reacts under an applied force (Bourne, 1978). In **paper V** the texturometer with a Kramer shear cell probe was used to evaluate the texture of cut lettuce, which was expressed as firmness. Firmness was defined as the maximum force to break the sample (Prakash et al., 2000; Han et al., 2004; Baur et al., 2005; **paper V**). Firmness is influenced by the structure of the vegetable, particularly cell shape and size, cell wall thickness and strength and the extent of cell to cell adhesion, alongside with the turgor (Toivonen and Brummel, 2008). These characteristics are highly related to the type of tissue (Toole et al., 2000). For instance, lettuce has two different types of tissue, photosynthetic and vascular (veins) (Martin-Diana et al., 2006). The vascular tissue has reinforcing fibers within the parenchyma which give a major contribution to the tissue strength (Tool et al., 2000).

In cut lettuce, loss of water and degradative process of cell wall by senescence are the main causes of loss of firmness (Prakash et al., 2000; Martin-Diana et al., 2006; Martinez

et al., 2007). Loss of water could be due to the lack of cuticle and sub-epidermal layers in the cut area (Wills et al., 1982; Toivonen and Brummell, 2008). The decrease in water in the tissue leads to a loss of turgor, reducing the firmness of cut lettuce (Prakash et al., 2000; Martin-Diana et al., 2006). Water loss can be reduced by packaging (Toivonen and Brummell, 2008). However, excessive accumulation of CO_2 in the packages has shown to enhance tissue softening in packaged cut lettuce in **paper V**. Firmness decreased through the storage time in packaged cut lettuce, being significantly lower in passive MAP F1 after 11 days of storage ($p \leq 0.05$). Probably, in packaged cut lettuce the extensive exposition to high CO_2 after 11 days of storage might have promoted loss of membrane compartmentalization. Loss of membrane compartmentalization might imply loss of turgor due to damage to cellular membrane (Faust et al., 1967). Hamza et al. (1996) also found that excessive accumulation of CO_2 in the packages enhance tissue softening in packaged romaine lettuce.

5.5.2. Soluble sugars and organic acids

Soluble sugars and organic acids are substrate for respiration in plant cells. Fructose, glucose and sucrose are the major soluble sugars in lettuce (Bolin and Huxsoll, 1991, Poulsen et al., 1991; Heimdal et al., 1995). Among the organic acids, malic acid is the major organic acid in lettuce followed by tartaric acid (Souci et al., 2000; Chandra et al., 2009; Flores et al., 2012, **paper V**). During storage of lettuce cut or intact, the content of soluble sugars and organic acids decrease as a consequence of respiration of the plant cell (Heimdal et al., 1995; Chandra et al., 2009, **paper V**). For instance, in cut lettuce stored in air was found a sharp drop in sucrose and malic acid probably in order to meet a higher respiratory demand than under passive MAPs (**paper V)**. To reduce the losses of sugars and organic acids in lettuce and other vegetables, MAP in combination with low storage temperatures is used, but extremely low O_2 and high CO_2 can trigger anaerobic reactions, as observed in **paper V**. Longer the storage under anaerobic conditions higher the loss of soluble sugars in packaged cut lettuce and presence of malolactic fermentation (**paper V**).

In addition, ascorbic acid has the capability to reduce *o*-quinones to *o*-diphenols, reducing the severity of browning (Cantos et al., 2001; Degl'Innocenti et al., 2005). There are a few studies regarding the action of ascorbic acid on browning in cut lettuce (Heimdal et al., 1995; Cantos et al., 2001). For instance, Heimdal et al. (1995) found that an inhibition of browning in cut lettuce packaged in moderate vacuum after 10 days of storage at 5 ºC might be caused by reduction of ascorbic acid to dehydroascorbic.

5.6. Analytical technique for the determination of soluble sugars, organic acids and chlorogenic acid using GC-MS

In paper V a simultaneous analysis of malic acid, tartaric acid, chlorogenic acid, ascorbic acid and soluble sugars such as glucose, fructose and sucrose was made using GC-MS. To be detected by GC-MS, these metabolites have to be converted to a volatile non polar and stable derivative form (Roessner et al., 2000). The most common used derivatization method for GC-MS involves the conversion of the original metabolite into their trimethylsilyl (TMS) or methoxime derivatives. In **paper V** trimethylsilyl derivatization was used (Roessner et al., 2000; Kanani and Klapa, 2007). To this end sugars and acids were exposed to silylation. The derivatization procedures imply to convert the OH group of the acid and carbohydrate molecule in an ether or ester group (Sparkman et al., 2011). Silylation reduces polarity, enhances volatility and thermal stability (Fluka chemie, 2012).

Liquid chromatography, capillary electrophoresis-MS and nuclear magnetic resonance are other analytical techniques used for simultaneous analysis of compounds under study in **paper V** (Kanani and Klapa, 2007). The advantage of GC-MS over these methods rely on a better separation of compounds in the gas phase than in liquid phase, high sensitivity that decrease the amount of the biological material needed for accurate measurements, and better identification power by MS due to extensive compound databases (Kanani et al., 2008).

5.7. Analysis of TMS derivatives

When GC-MS is used the peak area of the derivative is proportional to the concentration of the original metabolite. However, some biases can distort the proportionality relationship of the original metabolite concentration and the peak area of the metabolite derivative (Kanani and Klapa, 2007). The reasons are that a) some metabolites form more than one derivative and b) derivatization reaction has not been completed (Gehrke and Leimer, 1971; Kanani and Klapa, 2007). Therefore, experiments regarding the time of derivatization for all the metabolites under study in **paper V** were done.

In a solution glucose and fructose exist as a mixture of anomeric and acyclic forms. Derivatizations lead to four isomers for fructose and two for glucose. Each isomer has a peak in the GC-MS profile, as observed in Fig. 17 (Bradbury, 1990). Whereas, malic acid, tartaric acid, ascorbic acid, chlorogenic acid and sucrose formed only one peak. To evaluate this data a similar approach as presented by Kanani and Klapa (2007) and Kanani et al. (2008) was undertaken in **paper V**. In the case of malic acid, tartaric acid, ascorbic acid and chlorogenic acid the only peak is proportional to the concentration of the metabolite in the sample. However, for glucose, both TMS isomer peaks are proportional to the concentration of the metabolite in the sample. Thus, the largest one of these was chosen. For fructose, which formed four isomers, the largest one is no longer proportional to the concentration of the metabolite, as such all isomers peaks was summed up to maintain the proportionality relationship between the derivative and the original metabolite concentration.

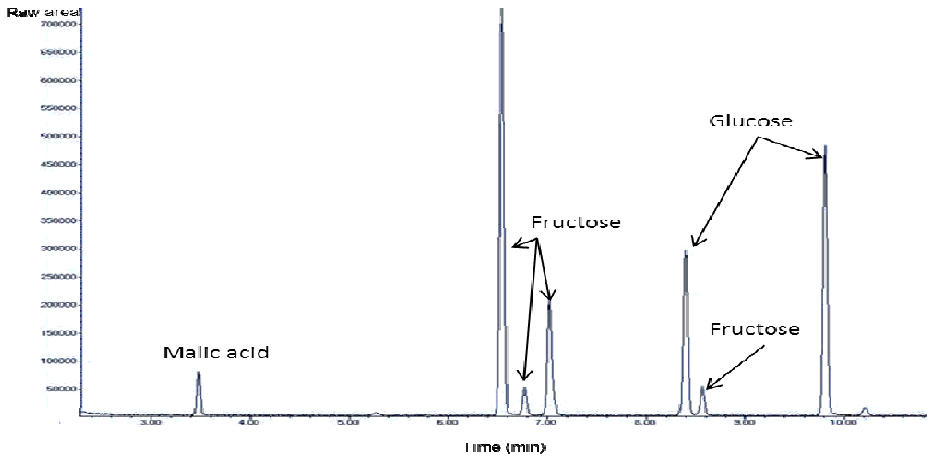


Figure 17. Total ion chromatogram (GC-MS) of glucose, fructose and malic acid as their TMS derivate of cut lettuce.

5.8. Influence of important factors in the formation of browning and other physicochemical characteristics in cut lettuce

***Paper V**: Changes in physicochemical characteristics of packaged and air stored cut iceberg lettuce upon storage and season*

In **paper V** iceberg lettuce of cultivars Platinas and Morinas was harvested in June, July, August and September 2009. Once lettuces were harvested, they were minimally processed and packaged in two passive modified atmospheres (MAP) built up by films of different permeabilities, F1 (OPALEN 65 AF) and F2 (OPP/PE-L 2040 AF), and storage in air. All packages were stored at 5°C. GC-MS was used for the analysis of glucose, fructose, sucrose, malic acid, tartaric acid, chlorogenic acid and ascorbic acid. Browning was evaluated using images taken with a scanner and subjected to color correction and thresholding. PPO activity and texture were also evaluated. The analyses were taken at 1, 5, 8 and 11 days of storage for packaged lettuce and at 1 and 5 days of storage for air stored samples

From the score plot of Fig. 18, it can be seen that June and August was clearly discriminated within season. From the loading plot it can be seen that in June most physicochemical characteristics had high values under anaerobic conditions. Differences in these values might be attributed to differences in climatic conditions within season. For instance, June 2009 was characterized with an average temperature of 13.5 ºC and more hours of sunshine (271 hours). It has been indicated that long period of photosynthetic activity in plants, results in increased production of photo assimilates such as carbohydrates (Taiz and Zeiger, 1998) and malic acid in grape berries (Hawker, 1969). Long days in June might explain the increase of soluble sugars and malic acid observed in packaged cut lettuce. The accumulation of carbohydrates could also contribute to changes in cell wall components, which are major contributors to firmness in lettuce (Martin-Diana et al., 2006; Toivonen and Brummell, 2008).

Among physicochemical characteristics, concentrations of sucrose and malic acid descreased as storage time increased (Fig.19). The drop in sucrose and malic acid in air storage samples was probably in order to meet a higher respiratory demanded than under passive MAPs. Respiration rate of cut fruits and vegetables is reduced under low O_2 and high CO_2 (Kader, 1986), but extremely low O_2 and high CO_2 can trigger anaerobic conditions, as observed in passive MAPs. Longer the storage under anaerobic conditions higher the loss of sucrose and reduction of malic acid, probably as a consequence of malolactic fermentation by lactic acid bacteria mainly found in passive MAP F1 after 11 days of storage ($p \leq 0.05$) (Cabrita et al., 2008). The extensive exposition to high CO_2 in passive MAP F1 also promoted loss of firmness (Fig.19) and decrease in pH. High CO_2 cause loss of membrane compartmentalization, which might have implied loss of turgor due to damage to cellular membrane (Faust, 1967).

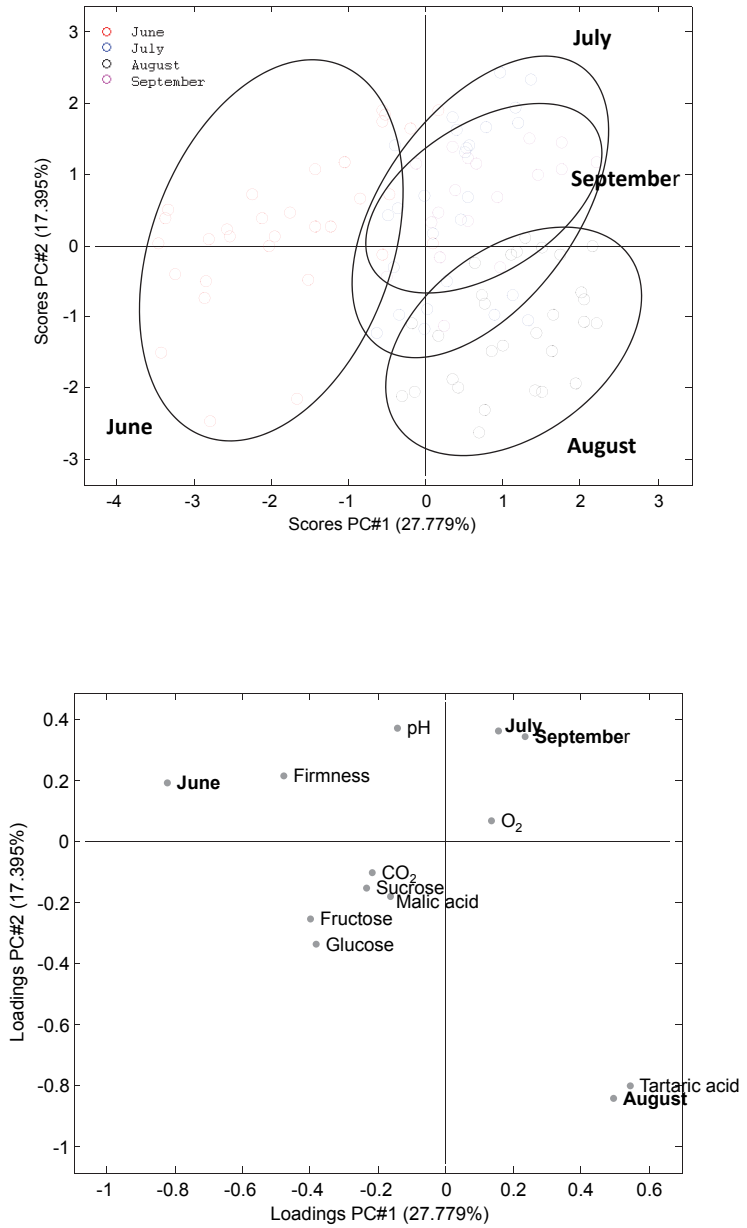
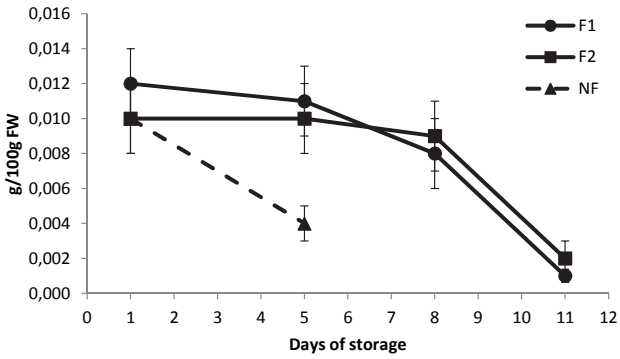
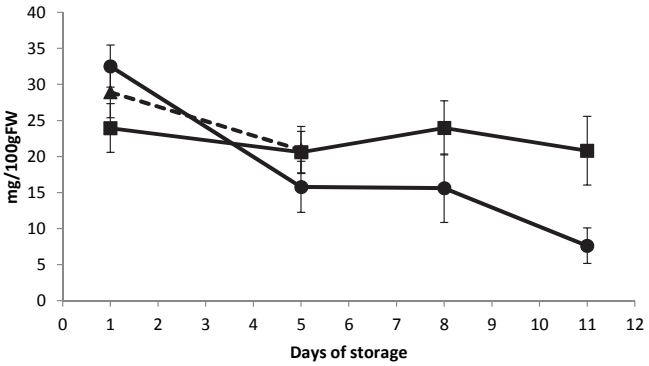


Figure 18. PLS-DA PLS-DA model for gas composition and physicochemical characteristics of cut lettuce cultivar Platinas and Morinas packaged in passive MAPs for up to 11 days and stored in air for 5 days at 5 ºC in June, July, August and September 2009.

a) Sucrose



b) Malic acid



c) Firmness

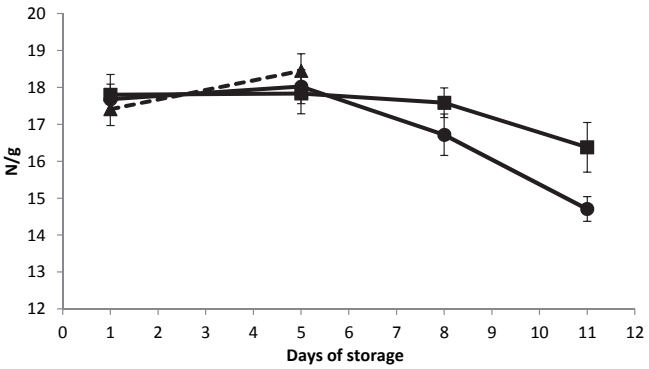


Figure 19. Changes in the concentration of selected soluble sugars, organic acids and firmness of cut lettuce stored for up to 11 days in passive MAP F1 and F2 and for 5 days in air at 5 ºC. Vertical lines represent the standard error of the mean (n=15).

Browning has been indicated to be the main limitation of the shelf-life of cut lettuce (Heimdal et al., 1995). As expected, the browning area (BA) increased in air stored samples as the time of storage increased. Browning was not found at 1 day of storage, but cut lettuce gradually turned brown during 5 days of storage. Cut lettuce stored in air presented favorable conditions for the development of browning. In air stored samples the concentration of chlorogenic acid was significantly higher than in packaged samples ($p \leq 0.05$) and PPO activity remained constant after 5 days of storage ($p \geq 0.05$).

On the contrary, browning was not observed in packaged samples during the storage time. Probably the inhibition of browning was a consequence of extremely low O_2 content and high CO_2 (Heimdal et al., 1995; Smyth et al., 1998). The results indicated that chlorogenic acid decreased as storage time increased in passive MAPs samples ($p \leq 0.05$), and PPO activity of cut lettuce packaged in passive MAP F2 sharply decrease after 1 day of storage ($p \leq 0.05$) (Table 9).

Table 9. Changes in ascorbic acid and chlorogenic acid of cut lettuce packaged in passive MAPs and PPO activity of passive MAP F2 as storage time increase.

Storage time (Days)	Ascorbic acid (mg/gFW)	Chlorogenic acid (mg/gFW)	PPO activity (U ml^{-1})
1	25.28 ± 23.84 (17) ^{b}	53.57 ± 38.17 (17) ^{a}	0.28 ± 0.10 (11)^{b}
5	7.61 ± 7.26 (20) ^{a}	30.14 ± 45.34 (20) ^{ab}	0.15 ± 0.08 (5)^{a}
8	10.37 ± 10.16 (25) ^{a}	63.49 ± 65.88 (25) ^{b}	0.16 ± 0.06 (5) ^{a}
11	2.43 ± 3.09 (16) ^{a}	15.33 ± 17.18 (16) ^{a}	

Data expressed as mean±standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$. Abbreviations: FW= fresh weight.

Season and cultivar are important factors for the development of browning (Matheis, 1983). Analysis of variance showed that there was more potential of browning in September and August than the rest of months ($p \leq 0.05$) and more for cultivar Platinas than Morinas ($p \leq 0.05$) (Table 10). Therefore a packaging to build up low O_2 and high CO_2 is of importance to control browning independent of the season and cultivar, as we proved in this study by the fact of browning was not observed in packaged samples.

However, there is a risk for the formation of off-odors (**paper IV**), tissue softening, decreased of sugars and malolactic fermentation, mainly in passive MAP F1after 11 days of storage.

Table 10. Brown area fraction (BA) of two cultivars of cut lettuce stored in air at 5 ºC during season 2009.

Factors	BA(%)
Cultivars	
Morinas	9.0 ± 4.5 (8) [a]
Platinas	13.0 ± 4.8 (6) [b]
Season 2009	
June	7.0 ± 1.3 (2) [ab]
July	6.0 ±3.2 (4) [a]
August	12.0 ±0.7 (4) [bc]
September	15.0±5.3 (4) [c]

Data expressed as mean±standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$.

Chapter 6. Conclusions and Perspectives

Conclusions

- This PhD thesis provides a better understanding of changes of volatile compounds, fructose, glucose, sucrose, malic acid, chlorogenic acid, tartaric acid, ascorbic acid, firmness, browning and respiration rate, as a part of quality changes induced by season, cultivar, packaging, storage time, storage temperature and method of preparation.
- Respiration rate was not a good an indicator of stress by cutting direction
- Transversal cutting was a more severe method of preparation than longitudinal cutting based on the increase in the levels of volatiles produced through the LOX pathway.
- Respiration rate was mainly affected by temperature of storage and season.
- A total of 52 volatile compounds were identified in this PhD project and of these 21 were shown to potent odorants of cut lettuce.
- Among the potent odorants, elemene, caryophyllene, β-selinene and 2,3-butanedione, enhanced under extremely low O_2 and high CO_2 built up in passive MAP F1 and likely to be off-odours.
- In August high production of this odorants was found and probably compromised the quality in terms of odour.
- Regarding the cultivars, Morinas and Diamantinas produce a significant amount of the undesirable odorant 2,3-butanedione.
- Browning was higher in August and September in samples stored under air.
- Browning was higher in cultivar Platinas in air stored samples.
- In June soluble sugars, malic acid and firmness were kept high under anaerobic conditions
- Browning was remarkably controlled in both passive MAPs due to extremely low O_2 and high CO_2 conditions; as a result, a product with good appearance was obtained.

However the increase of potent odorants likely to be off-odours were a limiting factor for shelf-life of packaged cut lettuce.

- Cut lettuce packed in passive MAP F1 after 11 days of storage showed a worse overall quality than MAP F2 due to tissue softening, decrease of sugars, malolactic fermentation and enhanced the production of odorants likely to be off-odours.
- The passive MAP built with film F2 seemed to be the most promising packaging.
- Storage of packaged cut lettuce for up to 11 days should be avoided.
- In this thesis was demonstrated that image analysis is a technique that allows an accurate quantification of browning by thresholding the colour corrected images.
- GC-MS was demonstrated to be a powerful tool for the identification and quantification of soluble sugars, organic acids, chlorogenic acid and ascorbic acid in cut lettuce.

Perspectives- Recommendations for future research

- To characterize the relationship between cutting, washing and drying with physiological and quality attributes of lettuce or other vegetables.
- To develop a sensory analysis for the determination of the shelf-life of cut lettuce in passive MAP F2.
- Industrial implementation of image analysis technique developed in this study as an automatic tool to asses browning and other color related characteristics of vegetables and fruits, i.e as a quality control procedure.
- In future works is suggested to investigate the influence of microbial growth in the formation of volatiles in cut lettuce.
- A more comprehensive study in lettuce volatiles and physicochemical constituents of cut lettuce covering at least two seasons is needed.
- Extend the application of GC-MS for the analysis of non-volatiles in other vegetables.

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Paper I

Image analysis for measuring enzymatic browning in minimally processed lettuce

Deza-Durand, K.M., Petersen, M.A., Poll, L., Larsen, M.

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Image Analysis for Measuring Enzymatic Browning in Minimally Processed Lettuce

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KEYWORDS
Enzymatic browning, Minimally processed lettuce, Colour correction, Thresholding, Scanner

SUMMARY
This study describes a novel method for the measurement of enzymatic browning in minimally processed lettuce that use a desktop flatbed scanner for image acquisition, colour dye patches for colour correction, and colour thresholding to quantify brown area fraction. Cut lettuce was stored at 5°C for 6 days and plus 1 day at room temperature (day 7). Changes in browning were assessed at 2, 6 and 7 days of storage using image analysis. The result showed an increase in browning as time and temperature of storage increased. It is concluded that this methodology is a reliable technique for the measurement of browning in cut lettuce.

INTRODUCTION

Minimally processed lettuce is one of the most popular ready-to-use vegetables (Zhou *et al.*, 2004). Browning has been reported as the main limitation of minimally processed lettuce shelf-life (Heimdal *et al.,* 1995) and it is still a problem for this industry in Denmark. During cutting the disruption of the cell induces numerous physiological changes such as an increase in enzyme activity of phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO), which causes browning (Saltveit, 2003).

Objective measurements of browning in cut lettuce have been made using colorimeters (Heimdal *et al.,* 1995) however, it has been claimed to be less successful to obtain an accurate colour representation due to the point by point measurement (O'Sullivan *et al.,* 2003). For this reason, new objective techniques to obtain a reliable quantification of browning are necessary. Under this context, image analysis is a prominent choice which has been reported to be representative, consistent and cost effective (O'Sullivan *et al.,* 2003).

When images are captured changes in lighting conditions from cameras, scanners, etc could affect the colours in the image obtaining unreliable information for comparison. In order to avoid this problem, a colour correction need to be performed (Russ, 2007). Once images are colour corrected they can be used for further analysis. Thresholding an image allows selecting features of interest (Russ, 2007). The aim of this study is to develop a new technique based on thresholding colour corrected images to asses the browning in minimally processed lettuce.

MATERIALS AND METHODS

Two hundred grams of cut lettuce were weighed and placed in 1.6 liter glass jar and covered with a perforated film in order to allow an atmosphere similar to air and prevent shriveling. The sample was stored at 5°C for 6 days and after 6 days it was left at room temperature for 1 day in order to obtain a wide range of lettuce's colours. Images of cut lettuce were captured using a desktop flatbed scanner (HP ScanJet 6200C, Hewlett Packard) at 0, 2, 6 and 7th day (6 days at 5°C plus 1 day at room temperature) of storage. Image included a colour reference consisting of 20 colour patches made of dyes used for painting. The scanner was covered with a black lid in order to exclude surrounding light. All images were saved in an uncompressed format (TIFF).

For colour correction a transformation was sought to each image to bring the colours of the reference patches to match a reference image (the image after 0 days of storage was chosen as a reference). The mean RGB pixel values of each colour patch in the image to be corrected were used to create a 20x3 matrix P_I of pixel values with one row per patch. A least square linear transformation into the corresponding matrix P_R for the reference image was obtained as follows:

$$L= [P_R^T P_R]^{-1} P_R^T P_I$$

To obtain the corrected pixel value p_c for each pixel in the image we computed:

$$p_c=L*p$$

where p is the uncorrected RGB pixel value.

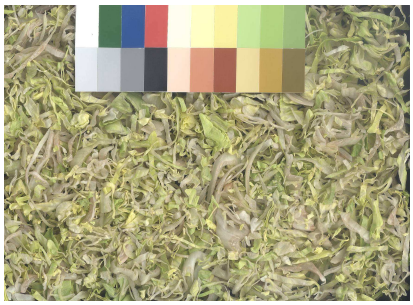
A thresholding to extract pixels of brown colour was applied to each corrected image. Images were first transferred from their original RGB representation to HSB (hue, saturation and brightness) where values range from 0-255 (Zhou, 2004; Russ, 2007). The area covered by the colour reference patches was masked out. Pixels from dark shade areas were removed by thresholding the brightness at 58% (removing pixels where $B \leq 147$). The number of remaining pixels is called the total number of pixels. Following this, the remaining pixels were thresheld with respect to hue (H) and saturation (S) retaining pixels where $H \leq 27$ or $H \geq 244$ (0 and 255 corresponding to the same red in the cylinder-shaped colour space) and $25\% < S \leq 100\%$. After thresholding, the brown area fraction (BA) was calculated as follows:

Brown area fraction= # brown pixels /#total pixels

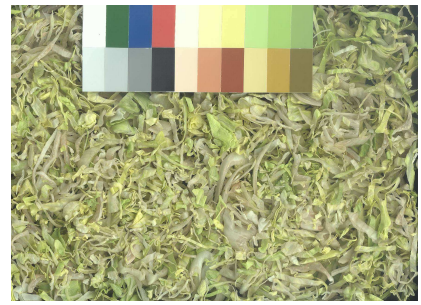
The ImageJ software (Abramoff *et al.,* 2004) was used for colour correction and thresholding of the images.

RESULTS AND DISCUSSIONS

Figure 1 shows the effect of colour correction. The scanner has only small variations in lighting (the diagonal of the transformation matrices were close to 1) but even small variations alter the colour recorded (Russ, 2007). Therefore, colour correction is an important step in order to obtain same colours to be compared and quantified. In this case (Fig.1), the corrected image shows a more clear definition of the colours than the original.



a) Original



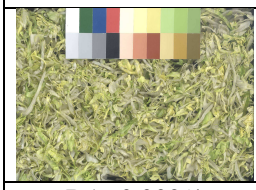
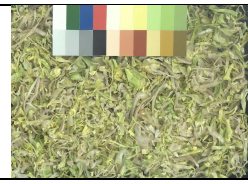
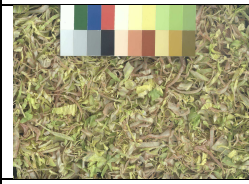
b) Corrected image

Fig.1. Colour correction of cut lettuce image at 2 days of storage at 5°C.

The colour reference was designed to span a range of colours which included those observed in cut lettuce, as well as clear and primary colors. Russ (2007) indicated that an adequate colour reference should cover a gamut of colours to be captured as well as the colours that we want to compare. In this study, the colour reference was also used as a reference for thresholding the browning, which facilitated the selection of brown pixels. Thresholding corrected images allowed the extraction and quantification of the same "browning". In the current research, browning was perceived as a pinking colour adjacent to the wound area. Thresholding is an essential step in order to get information from the image to perform further measurements (Sun, 2000).

It is important to mention that irregular shape of cut lettuce allows the formation of shade areas in the image. These areas were excluded for the analysis because they were too dark to get an accurate description of browning. In actual application, taking more than one picture per sample (mixing the sample before each capture) would yield a more complete representation at the lettuce surface.

Table 1. Brown area fraction (BA) of cut lettuce at different storage conditions.

0 days at 5 °C	2 days at 5 °C	6 days at 5 °C	Day 7 (6 days at 5°C plus 1 day at room temperature)
			
BA=0.003%	BA=0.15%	BA=10.27%	BA=15.23%

As expected, the browning increases as the time and temperature of storage increased (Table 1). Browning was hardly observed at 0 and after 2 days of storage with a BA below 1%. As storage time increased, the cut lettuce gradually turned brown, reaching a BA greater than 10% after 6 days of storage at 5°C. It seems that PAL induced the biosynthesis of polyphenols during storage, which were oxidized by PPO resulting of an increased browning rate in cut lettuce. Murata *et al* (2004) mentioned that browning in cut lettuce takes few days to be perceived due to an initial low amount of polyphenols. It has been reported that polyphenol content is the limiting factor of browning in cut lettuce (Murata *et al.,* 2004). Once cut lettuce was under temperature abuse (day 7) the BA was 1.5 time the BA value at 6

days of storage at 5 °C. The increase of browning can be attributed to an increase of enzymatic activity that allowed a rapid formation of brown pigments. Therefore, high temperatures of storage can shorten the shelf-life of cut-vegetables (Wills *et al.,* 1982). Our result showed that this technique can measure the browning in cut lettuce.

Moreover, visual judgment of brown area fraction may differ from the brown area fraction that was obtained with this technique. This may be attributed that visual inspection is not a quantitative process (Russ, 2007). It has been reported that visual inspection can be biased by features in the image (Russ, 2007); for example at day 7 the brown color was more saturated (easy to find difference) and more spread in the image, that could influence the visual judgment, obtaining a larger brown area fraction than the calculated. For this reason, it would be of interest in future experiments the use of human panel in order to correlate a subjective evaluation with the brown area fraction obtained with this technique.

CONCLUSIONS

It can be concluded from this small pilot study that this new technique can be used for measuring the browning in cut lettuce. The advantage of this method is that it provides a better representation of colour by the fact that the flatbed scanner captures a bigger area of the sample than colorimeters. In addition, this technique allows an accurate quantification of browning by thresholding the colour corrected images. Further experiments, which involve a sensory panel, are required in order to fully validate this technique as an effective tool for measuring the browning in minimally processed lettuce.

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Paper II

The effect of cutting direction on aroma compounds and respiration rate of fresh-cut iceberg lettuce (*Lactuca sativa* L.)

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The effect of cutting direction on aroma compounds and respiration rate of fresh-cut iceberg lettuce (*Lactuca sativa* L.)

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ABSTRACT

The purpose of this research was to investigate whether cutting direction and storage temperature could influence aroma formation and respiration rates in minimally processed lettuce. Lettuce was cut both longitudinally and transverse to the mid-rib and stored at 6 and 10 °C for 4 and 5 days. The experiment was performed in January and March 2008. Changes in respiration rate were analyzed during storage, and aroma analysis was carried out after 4 and 5 days of storage in January and March, respectively. Respiration rates increased with increasing storage temperatures. Transverse cuts to the rib were strongly related with volatiles of the lipoxygenase (LOX) pathway i.e. cis-3-hexenal, cis-3-hexenol and trans-2-hexenol, while longitudinal cutting enhanced formation of volatiles from other metabolic routes. Aroma formation was also influenced by storage temperature, where higher storage temperatures resulted in increases in α-longipinene, 2-methylbutanal and 3-methylbutanal. Our results demonstrate that cutting the lettuce transverse to the mid-rib caused more severe damage to the tissue than longitudinal cutting, based on aroma production of LOX volatiles.

1. Introduction

Iceberg lettuce (*Lactuca sativa* L.), cut or shredded, is considered one of the most popular of fresh-cut vegetables, and a growing demand for this convenient and time saving product has occurred in developed countries over the last few years (Ragaert et al., 2004). Preparation of fresh-cut lettuce (e.g. cutting, shredding) causes disruption of cells, which induces physiological responses such as an increase in respiration rate, ethylene and phenolic compounds and formation of volatiles (Saltveit, 2003; Belitz et al., 2004). Browning has been suggested as the main factor limitating shelf-life in fresh-cut lettuce (Heimdal et al., 1995), through enzymatic oxidation of phenolic compounds, but other factors such as loss of aroma and off-odours could also shorten storage life of the product. Allende et al. (2004) reported that fresh-cut "Lollo Rosso" lettuce after 7 days of storage at 5 °C showed a decline in general appearance and aroma, based on sensory analysis.

The loss of quality in fresh-cut lettuce needs to be minimized in order to extend the shelf-life of the product. Temperature is the most important environmental factor affecting metabolic reactions in commodities (Bolin and Huxsoll, 1991), and low temperature has been shown to reduce respiration rates and browning of fresh-cut vegetables. Cut or shredded iceberg lettuce has been recommended to be stored in a range from 0 to 5 °C (Kader and Saltveit, 2003a). However, temperature fluctuations along the supply chain may cause loss of the product quality.

Methods of preparation of fresh-cut lettuce which minimize cutting damage are highly desirable. Martinez et al. (2008) and Bolin and Huxsoll (1991) found that tearing lettuce led to a lower respiration rate and deterioration than cutting with a sharp knife or shredding due to less tissue damage. Likewise, cutting direction (longitudinal or transverse) could influence the wounding response of the commodity (Saltveit, 2003). Abe et al. (1998) indicated that transverse cutting of green tip bananas leads to lower respiration rates and delayed browning in comparison with longitudinal cutting.

Lettuce volatiles have received little attention, even though this commodity has great commercial value and volatiles are important parameters in assessing quality of lettuce (Smyth et al., 1998; Nielsen and Poll, 2006; Lonchamp et al., 2009). Once the plant tissue is disrupted, enzymatic reactions allow the formation of volatiles such as alcohols, aldehydes, terpenes, esters and acids (Belitz et al., 2004). Moreover, some volatile production is more specific to mechanical injury, such as the C6 aldehydes and alcohols from lipoxygenase (LOX) activity. cis-3-Hexenol and trans-2-hexenal are volatiles from the LOX pathway and are key aroma compounds of iceberg lettuce (Arey et al., 1991). Also, terpenes such as trans-α-bisabolene, α-copaene, valencene, germacrene, α-terpinolene, and α-longipinene, together with 2-methoxy-3-isobutylpyrazine, have been identified as important aroma compounds of iceberg lettuce

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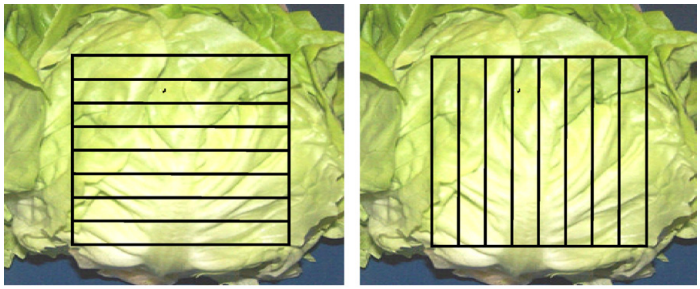

Fig. 1. Lettuce cut transverse and longitudinal to the mid-rib. Piece size was in the range of 10–12 mm wide and 8 cm long.

(Nielsen and Poll, 2006; Lonchamp et al., 2009). However, volatiles also contribute to the formation of off-odours, depending on their concentration; they can shorten the storage life of the product. Poll et al. (2006) stated that aldehydes in lower concentrations contribute to characteristic aromas in leeks but at higher concentrations can cause off-odours. Storage temperatures can also influence changes in aroma compounds in vegetables. Jacobsson et al. (2004) found that aroma compounds of broccoli increase their concentration at 10 °C in comparison to 4 °C in storage due to membrane deterioration.

The important effects of temperature and the direction of the cut on respiration and aroma compounds in fresh-cut lettuce have not been reported previously to our knowledge. Therefore, we aimed to investigate the respiration rate and aroma compounds of fresh-cut lettuce as a function of cutting direction and storage temperature. The outcomes of this research are relevant to the small food business, i.e. catering and restaurants, due to the possible better control on the cutting direction than occurs in industry.

2. Materials and methods

2.1. Plant material

Eight heads of Iceberg lettuce (*L. sativa* L.) of Spanish origin were purchased from a local market in Copenhagen, Denmark. The experiment was replicated in January and March 2008. The lettuces were of unknown cultivars in both replicates. Lettuces were taken to the laboratory and maturity was measured as firmness under hand pressure (Kader et al., 1973). Lettuces were held at 2 °C and processed the same day.

2.2. Sample preparation

Leaves with defects and the stems of each head were discarded. Lettuce leaves were cut longitudinally and transversely to the mid-rib, as indicated in Fig. 1. The sizes of the cut pieces were in the range of 10–12 mm wide and 8 cm long. 100 g of cut lettuce of each cutting direction were weighed and placed in a 720 mL glass jar and sealed with household film containing 40 punctured holes of 1 mm in diameter. The punctured film was used to allow an atmosphere similar to air inside the jars while still avoiding rapid shrivelling of the lettuce pieces. The jars were stored at 6 and 10 °C for 4 days in January 2008 and 5 days in March 2008, with three replicates for each treatment. The storage temperatures used were chosen to simulate market temperature conditions (6 °C) and fluctuations in the supply chain (10 °C).

2.3. Respiration rate

The respiration rate was measured every day until the end of the storage time. To measure CO_2 production, the film on the jars was replaced with a metal lid with a septum and kept sealed for between 2 and 5 h in order to obtain an accurate CO_2 measurement, above 0.5% as recommended by Kader and Saltveit (2003a). A syringe was inserted into the septum and the CO_2 was measured using a gas analyzer (Gaspace, Systech Instruments Ltd., Texas, USA). Carbon dioxide standards were used for calibration of the equipment. The respiration rate was calculated as mg CO_2 produced per kg lettuce per h on a fresh weight basis, as in the following equation according to Kader and Saltveit (2003a):

$$R_{CO_2} = \frac{\text{mg } CO_2}{\text{weight (kg)} \times \text{time (h)}}$$

At the end of the measurement the lid was removed and the punctured film was placed again on the top of the jar.

2.4. Dynamic headspace sampling

One hundred grams of fresh-cut lettuce (longitudinal or transverse) was blended with 100 mL of tap water and 2 mL of internal standard (50 μg mL^{-1}, 4 methyl-1-pentanol, Sigma–Aldrich) was added. The sample was homogenized for 15 s using a blender (Struers Kebo lab) and poured into a 1 L glass flask. The blender cup was washed with 50 mL of tap water and added to the suspension and the flask was closed with a purge head. The sample was temperature-equilibrated for 10 min in a water bath at 30 °C with magnetic stirring (200 rpm) and then purged with nitrogen (100 mL/min) for 25 min. The volatiles were trapped in a stainless steel trap containing 250 mg of Tenax-TA, mesh size 60/80 and density 0.37 g mL^{-1} (Buchem bv, Apeldoorn, The Netherlands).

2.5. Gas chromatography (GC)–mass spectrometry (MS)

The volatiles collected in the traps were thermally desorbed using an automatic thermal desorption device (ATD 400, Perkin Elmer, Norwalk, USA). Traps were desorbed by heating to 250 °C with helium flow of 60 mL min^{-1} for 15 min and volatiles collected in a cold trap which subsequently was flash-heated to 300 °C and held for 4 min. A split ratio of 1:10 was applied to transfer the volatiles to a GC–MS for separation and identification. The GC–MS used was a G1800 GCD System (Hewlett-Packard, Palo Alto, CA, USA) equipped with a DB-Wax capillary column (30 m × 25 mm × 0.25 μm) (J&W Scientific). The column flow rate was 1.0 mL min^{-1} using helium as a carrier gas. The temperature of the column was held at 45 °C for 10 min and then increased by 6 °C min^{-1} up to 240 °C, which was kept constant for 10 min.

The mass spectrometric detector operated in the electron ionization mode and scanned mass/charge (m/z) between 15 and 300. All volatile identification was made by matching obtained mass spectra with those in the G1035A Wiley library (Hewlett-Packard, Palo Alto, CA, USA) and by comparison of the retention time and mass spectra with those of authentic reference standards. Volatile peak area was calculated on the basis of single ions and their concentration expressed as relative area by dividing the peak area of the volatile by the area of the internal standard.

2.6. Statistical analysis

Respiration rate data were analyzed as a split–plot design for repeated measurements with a factorial arrangement, in which the factors were the direction of the cut, temperature of storage, storage time and the experiment replications. A 3-way ANOVA was used for the aroma data analysis with direction of the cut, temperature of storage and the experiment replications as factors. The difference between means was determined by Tukey's multiple range test at a 95% confidence interval. The statistical analysis was performed with InfoStat Statistical Software (Di Rienzo et al., 2008). Principal component analysis (PCA) was also carried to determine the variations in the aroma compounds using Latentix Ver. 2.00 (Latent5, Copenhagen, Denmark, www.latentix.com).

3. Results and discussion

3.1. Respiration rate

Respiration rate gives an immediate overview of the metabolism of a commodity, where higher respiration is an indicator of shorter shelf-life (Kader and Saltveit, 2003a). Lettuce has been classified as a commodity with a moderate respiration rate (Kader, 2002), and wounding the tissue by cutting speeds up the respiration rate.

A significantly higher respiration rate of lettuce was observed for transverse cutting after 0 days of storage in comparison with longitudinal cutting ($p \leq 0.05$), at both storage temperatures. It is known that cutting the tissue of commodities can cause an immediate increase in respiration rate due to enhanced aerobic mitochondrial respiration by enzymes such as phosphofructokinase and cytochrome oxidase (Asahi, 1978). However, in both replications of the experiment, this higher increase in respiration rate was transitory due to the fact that respiration had decreased sharply after 1 day of storage (Fig. 2). Toivonen and DeEll (2002) have noted that the increase in respiration rate after cutting is observed until a normal aerobic respiration is reestablished, which depends on the commodity.

Analysis of variance showed significantly higher respiration rates at 10 °C than at 6 °C in storage ($p \leq 0.05$), as shown in Table 1. Higher storage temperatures are expected to increase the respiration rate due to increased reaction rates of many pathways in cell respiration (Wills et al., 1982; Watada and Qi, 1999). When comparing the replications of the experiment, data from January showed significantly higher mean values of respiration rates than that from March ($p \leq 0.05$; Table 1). This could be attributed to differences in maturity state. Lettuce heads bought in January were mature, whereas those from March were over-mature, which could explain the trend to higher respiration rates in January due to young cells being more active than old cells (Wills et al., 1982; Kays, 1991). Likewise, lettuce heads used in this experiment were of Spanish origin but of an unknown cultivar, and it is possible that use of different cultivars leads to the differences in CO_2 production (Kays, 1991).

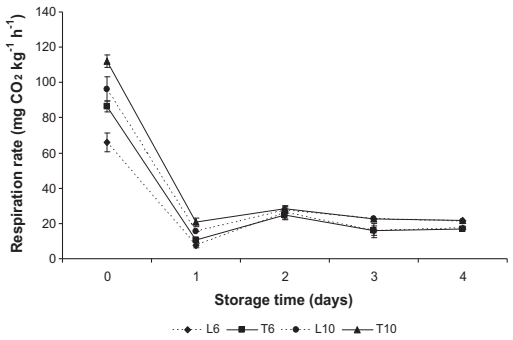

Fig. 2. Respiration rate of iceberg lettuce cut transverse and longitudinal to the mid-rib stored at 6 and 10 °C for 5 days in March 2008. The symbols are the average of three replicates and the vertical lines represent the standard deviation of the replicates. *Abbreviations*: L6, lettuce cut longitudinal stored at 6 °C; T6, lettuce cut transverse stored at 6 °C; L10, lettuce cut longitudinal stored at 10 °C; T10, lettuce cut transverse stored at 10 °C.

Previous findings indicate that the direction of the cut affects the wounding response of minimally processed vegetables, as shown in this study. It seems that the response due to the direction of the cut depends on species and severity of tissue damage (Brecht, 1995). For instance, for green tip bananas the increase in respiration rate and deterioration was higher in longitudinally cut sections than in transverse ones due to size of the cut surface (Abe et al., 1998), whereas for green bell peppers cut lengthwise, cutting resulted in a higher rate of deterioration, probably related to solubilization of pectin on the cut surface (Zhou et al., 1992). In this study, respiration rate of transverse cut samples was higher than longitudinally cut ones. This may be due to increased cutting surface area and damage to more cells of the mid-rib. A lettuce leaf is a complex material composed of photosynthetic and vascular tissues, and inner leaves differ metabolically from outer leaves in the same lettuce head (Toole et al., 2000). Due to lettuce complexity, care was taken to obtain a uniform sample. The increase in respiration rate by transverse cutting was temporary since it was observed mainly at 0 days of storage, as shown in Fig. 2. In the current study, respiration rate may not be considered as an indicator of the effect of cutting direction, since respiration rate after the first day became largely constant. But the response to wounding could extend beyond the cut surface, allowing accumulation of secondary metabolites. Therefore, if transverse cutting damages more cells, it could promote an increase in lipid peroxidation and membrane deterioration more than longitudinal cutting. Membrane deterioration could allow the oxidation of linolenic and linoleic fatty acids catalysed by lipoxygenase (LOX) in the presence of oxygen, gen-

Table 1
Respiration rate of fresh-cut lettuce prepared with different cutting directions and stored at 6 and 10 °C in January and March 2008.

Factors	Respiration rate (mg CO_2 kg^{-1} h^{-1})
Cutting	
Transverse	39.3 ± 29.5 (47)[b]
Longitudinal	35.1 ± 25.0 (47)[a]
Temperature of storage	
10 °C	43.0 ± 30.8 (46)[b]
6 °C	31.7 ± 22.4 (48)[a]
Replicates of the experiments	
January 2008	41.9 ± 21.1 (36)[b]
March 2008	34.3 ± 30.3 (58)[a]

Data expressed as mean ± standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$.

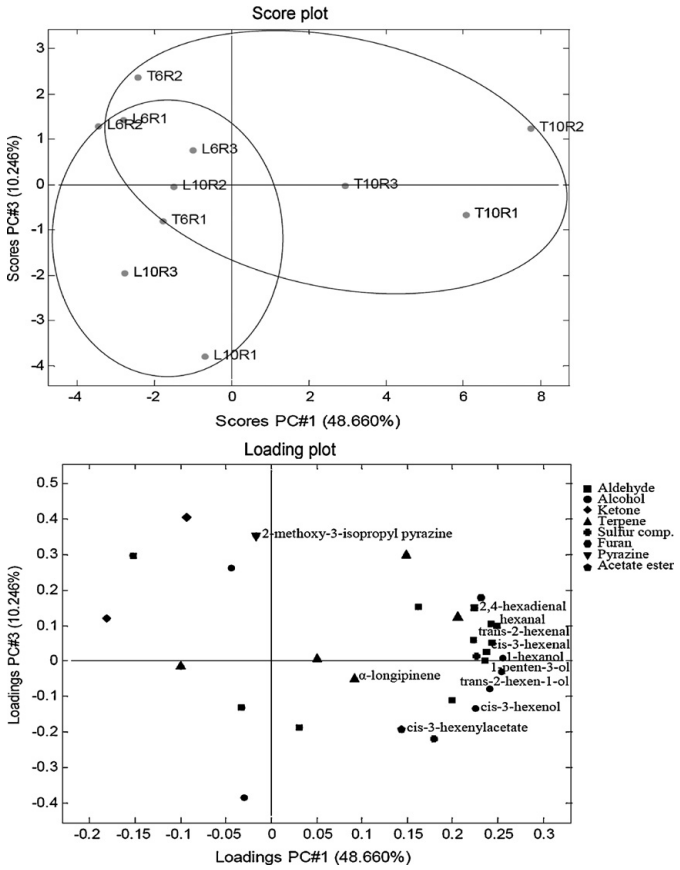

Fig. 3. PCA of 30 volatile compounds found in lettuce cut transverse and longitudinal after 4 days of storage in air at 6 and 10 °C in January 2008. *Abbreviations*: T6, lettuce cut transverse stored at 6 °C; L6, lettuce cut longitudinal stored at 6 °C; T10, lettuce cut transverse stored at 10 °C; L10, lettuce cut longitudinal stored at 10 °C; replicates = R1, R2, R3.

erating hydroperoxides that could lead to the formation of odour compounds such as C6 aldehydes (Belitz et al., 2004), as will be seen in Section 3.2.

3.2. Aroma compounds

Forty five compounds were identified in the current study. The aroma compounds were mainly aldehydes (14), alcohols (12), terpenes (5), ketones (4) acids (4), sulfur compounds (2), acetate esters (2), pyrazine (1) and furan (1) (Table 2). When comparing replicates of the experiment, March samples had a higher number of aroma compounds (41) than January ones (30). The difference between the two replicates could be attributed to differences in maturity of lettuce bought in January and March 2008. Furthermore, other factors such as cultivar, preharvest and environmental conditions may also affect the production of different volatiles between replicates of the experiment. Unfortunately, lettuce heads used in this study were of unknown cultivars in both replicates of the experiment, and preharvest and environmental conditions were out of the scope of this study. For instance in corn, cis-3-hexenyl acetate significantly decreases with an increase in light and soil humidity (Gouinguene and Turlings, 2002).

The effect of the cutting direction on the aroma compounds of lettuce has not been studied before to our knowledge. An analysis using PCA was carried out on 30 and 41 compounds from the January and March samples respectively. The analysis was focused on volatiles from the LOX pathway because those aroma compounds are related to disrupted tissue, as previously reported in tomatoes (Baldwin et al., 2000) and lettuce (Arey et al., 1991), including cis-3-hexanal, cis-3-hexenol, cis-3-hexenyl acetate, trans-2-hexenal, trans-2-hexen-1-ol, hexanal, hexenol, 2,4-hexadienal and 1-penten-3-ol. α-Longipinene and 2-methoxy-3-isopropylpyrazine were also selected based on their importance as key odour compounds in lettuce (Nielsen and Poll, 2006; Lonchamp et al., 2009).

Fig. 3 shows the score and loadings plots of data from January. The first principal component (PC1) clearly discriminated the transverse cutting at 10 °C. PC1 and PC3 in combination separated storage temperatures, explaining 49% and 10% of the variance, respectively. Similar trends were observed in the March data. PC1 (61%) discriminated the directions of the cutting, but only at 6 °C; no separation was achieved at 10 °C of storage, whereas PC2 (11%) mainly separated storage temperatures (Fig. 4). In the January samples, volatiles from the LOX pathway seemed to be strongly related to the transverse cut at the high storage temperature.

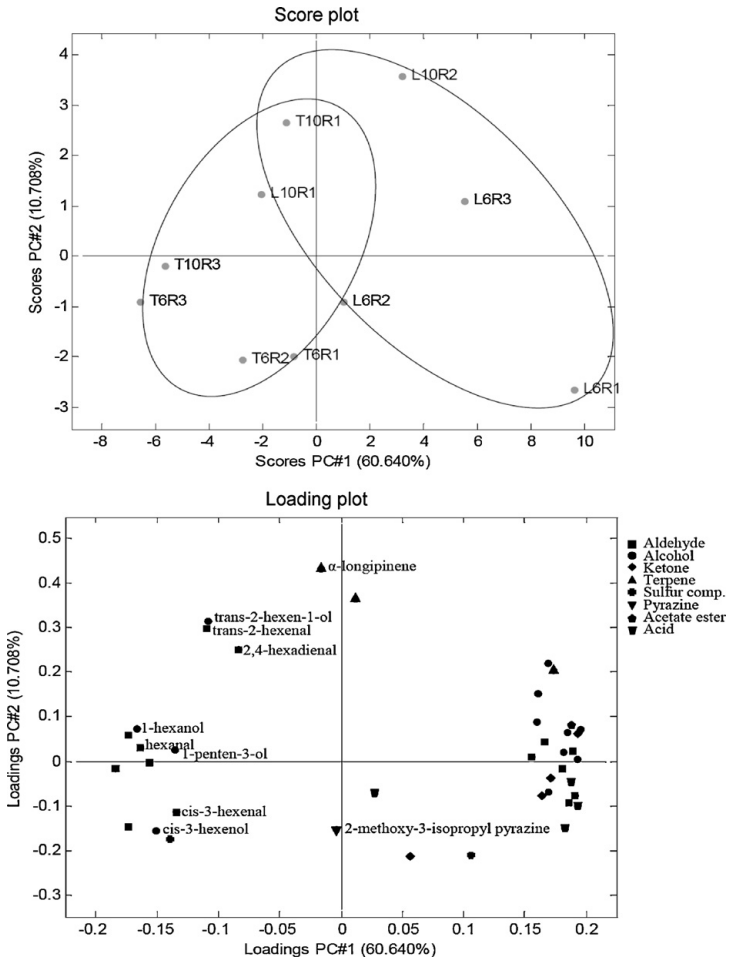

Fig. 4. PCA of 41 volatile compounds found in lettuce cut transverse and longitudinal after 5 days of storage in air at 6 and 10 °C in March 2008. *Abbreviations*: T6, lettuce cut transverse stored at 6 °C; L6, lettuce cut longitudinal stored at 6 °C; T10, lettuce cut transverse stored at 10 °C; L10, lettuce cut longitudinal stored at 10 °C; replicates = R1, R2, R3.

Furthermore, in the March data, cis-3-hexenal and cis-3-hexenol were also strongly associated with transverse cutting but at 6 °C, while trans-2-hexenal, trans-2-hexen-1-ol, 2-ethyl-1-hexanol, 2,4-hexadienal, hexanal, 1-hexenol and 1-penten-3-ol seemed to be related to higher storage temperatures. Likewise, 2-methoxy-3-isopropylpyrazine and α-longipinene were strongly related with 6 and 10 °C in both experiments.

The PCA was confirmed by three-way ANOVA. The relative areas of cis-3-hexenal, cis-3-hexenol and trans-2-hexen-1-ol were significantly higher in lettuce cut transversely ($p \leq 0.05$) than longitudinally (Fig. 5). LOX has been shown to be a stress-related enzyme (Hildebrand, 1989), and an increase in these compounds by cutting the lettuce in the transverse direction might indicate a greater disruption of membranes by this method of preparation. For instance, an increase in cis-3-hexenol in packaged broccoli stored at 4 °C is an indicator of membrane breakdown and senescence (Jacobsson et al., 2004). Likewise, volatiles of the LOX pathway, particularly 1-penten-3-ol, hexanal, hexanol, 2,4-hexadienal and trans-2-hexenal, were found to be significantly higher when lettuce were cut transversely and stored at 10 °C in January 2008 ($p \leq 0.05$). Among the compounds, trans-2-hexenal was the most affected. It increased up to 10 times more than other LOX volatiles (Table 3). It seems that the increase in these compounds was also affected by the higher storage temperature, resulting in an increase in membrane deterioration from increasing activity of enzymes related to senescence and lipid degradation such as acyl hydrolase and LOX. An increase in the activity of acyl hydrolase would speed the release of linoleic and linolenic acids, substrates for LOX under oxidative conditions (Paliyath and Droillard, 1992). Maturity of the lettuce in January also had an effect on the production of LOX volatiles transversely cut. In January, lettuce heads were mature, which means that LOX activity might be high in comparison with overmature lettuce found in March. Matsui et al. (1997) showed that LOX activity is reduced with an increase in maturity. High LOX activity would infer faster reaction with substrates released by severe deterioration of membranes caused by transverse cutting, together

Table 2
Aroma compounds of fresh-cut lettuce stored at 6 and 10 °C in January and March 2008.

Aroma compounds	
Aldehydes	*Terpenes*
2-Methylpropanal[a]	β-Elemene[b]
2-Propenal[a,c]	β-Caryophyllene[b,c]
2-Methylbutanal[a,c]	α-Longipinene[a]
3-Methylbutanal[a,c]	α-Muurolene[a]
Pentanal[c,d]	Limonene[a,c]
Hexanal[a,c]	*Ketones*
Cis-3-hexenal[a]	2-Butanone[d]
Heptanal[c,d]	Acetophenone[d]
Trans-2-hexenal[a,c]	Geranylacetone[a,c]
Octanal[a,c]	6-Methyl-5-hepten-2-one[a,c]
Nonanal[a,c]	*Acids*
Decanal[a]	Propanoic acid[c,d]
Benzaldehyde[a,c]	Butanoic acid[c,d]
2,4-Hexadienal[a,c]	Hexanoic acid[c,d]
Alcohols	2-Methyl butyric acid[d]
2-Methyl-1-propanol[a,c]	*Sulfur compounds*
1-Butanol[c,d]	Dimethyl sulfide[a,c]
1-Penten-3-ol[a,c]	Dimethyl sulfoxide[a]
1-Pentanol[c,d]	*Acetate esters*
1-Hexanol[a,c]	Ethyl acetate[c,d]
Cis-3-hexenol[a]	Cis-3-hexenyl acetate[b]
Trans-2-hexen-1-ol[a,c]	*Pyrazine*
2-Ethyl-1-hexanol[a]	2-Methoxy-3-isopropyl pyrazine[a]
1,2-Methoxypropoxy-2-propanol[c,d]	*Furan*
1-Octanol[c,d]	2-Ethylfuran[b,c]
Nonanol[c,d]	
Phenol[d]	

[a] Aroma compound found in January and March 2008.
[b] Aroma compound found only in January 2008.
[c] Mass spectra agreed with authentic standards and Wiley library.
[d] Aroma compound found only in March 2008.

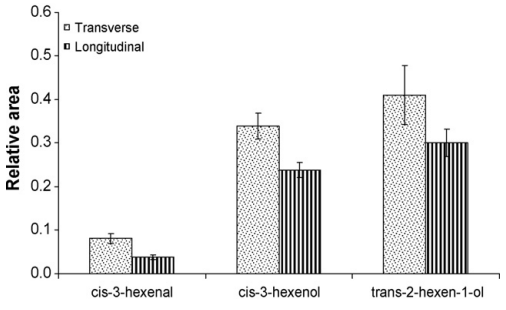

Fig. 5. Changes in cis-3-hexenal, cis-3-hexenol and trans-2-hexen-1-ol of lettuce cut transverse and longitudinal to the mid-rib. Vertical lines represent the standard error of the mean ($n = 10$).

with a high storage temperature enhancing the formation of LOX volatiles. Membrane deterioration has been identified as enhancing the volatiles from the LOX pathway (Chin and Lindsay, 1993; Charron and Cantliffe, 1995).

Aroma of lettuce has been characterized by LOX volatiles such as cis-3-hexenal, cis-3-hexenol and trans-2-hexenal (Arey et al., 1991; Charron et al., 1996). The increase of these aroma compounds by transverse cutting associated with greater deterioration of lettuce tissue may have caused the formation of off-odours since they are present in higher concentrations (Poll et al., 2006). Unfortunately a sensory analysis of the aroma was not carried out in this study and the changes found in these compounds cannot be related to human acceptability of the product. For this reason, a sensory analysis of the aroma of lettuce should be included in future studies to know if the effect of cutting is perceived by the consumers.

Longitudinal cutting of lettuce significantly increased aroma compounds from other enzymatic reactions. In January and March samples, 6-methyl-5-hepten-2-one was significantly higher than in transversely cut samples ($p \leq 0.05$). 6-Methyl-5-hepten-2-one is formed by oxidation of β-carotene, and β-carotene is the main carotenoid and precursor of vitamin A in lettuce (Baldwin et al., 2000; Sobolev et al., 2005). 2-Butanone, propanoic acid, butanoic acid, 1-butanol, 1-pentanol, 1-octanol, phenol, 1,2-methoxypropoxy-2-propanol, ethyl acetate, nonanal, decanal, benzaldehyde and limonene were significantly higher in longitudinally cut samples and only identified in the March samples ($p \leq 0.05$), with the exception of the four latter compounds observed in both replicates of the experiment (Table 4). Those compounds might arise from different metabolic routes, i.e. acids are released from vacuoles by free radicals produced from LOX activity (Omarkhayyam, 1986), and alcohols such as 1-butanol, 1-pentanol, 1-octanol and phenol could be reduced from their respective aldehydes by alcohol dehydrogenase (Toivonen, 1997), whereas nonanal and decanal could be formed by hydroperoxide cleavage of unsaturated fatty acids of lettuce. It seems that the damage caused to the tissue by the longitudinal cutting differed from the transverse cutting, leading to differences in volatile formation. Moreover, the diversity of volatiles observed in the March samples from the longitudinal cutting could also be influenced by the stage of maturity. In over mature lettuce, LOX activity decreases (Matsui et al., 1997), which may lead to reduction of formation of LOX volatiles and possibly an increase in the formation of aroma compounds from other metabolic routes such as aliphatic aldehydes and alcohols as part of senescence in over mature lettuce. Further research is needed on the metabolic routes for volatile formation in lettuce and their relation to severity of tissue damage and/or to different parts of lettuce that could have different metabolic behavior i.e. inner and outer leaves.

Storage temperature can also influence the production of individual volatile compounds of lettuce. α-Longipinene, 2-methylbutanal and 3-methylbutanal were at significantly higher

Table 3
Effect of experiment, storage temperature and type of cutting on the relative area of aroma compounds of lettuce stored in air.

Aroma compounds	Experiment							
	January 2008				March 2008			
	6 °C		10 °C		6 °C		10 °C	
	T	L	T	L	T	L	T	L
Trans-2-hexenal	0.83^{a}	0.78^{a}	3.16^{b}	0.71^{a}	0.27^{a}	0.19^{a}	0.48^{a}	0.56^{a}
2,4-Hexadienal	0.03^{d}	0.03^{cd}	0.07^{e}	0.03^{bcd}	0.01^{ab}	0.0028^{a}	0.02^{abc}	0.04^{d}
Hexanal	0.13^{a}	0.11^{a}	0.58^{b}	0.12^{a}	0.15^{a}	0.07^{a}	0.15^{a}	0.11^{b}
Hexanol	0.31^{a}	0.24^{a}	0.79^{b}	0.29^{a}	0.26^{a}	0.15^{a}	0.28^{a}	0.21^{a}
1-Penten-3-ol	0.05^{a}	0.05^{a}	0.08^{b}	0.05^{a}	0.04^{a}	0.03^{a}	0.04^{a}	0.04^{a}

Values with different letters across a row are significantly different ($p \leq 0.05$). *Abbreviations*: T = transverse cut; L = longitudinal cut.

Table 4
Interaction between experiment and type of cutting on the relative area of aroma compounds of lettuce stored in air for 4 and 5 days in January and March.

Aroma compounds	Experiment			
	January 2008		March 2008	
	Transverse cutting	Longitudinal cutting	Transverse cutting	Longitudinal cutting
2-Butanone	0^{a}	0^{a}	0.03^{a}	0.06^{b}
1-Butanol	0^{a}	0^{a}	0.01^{a}	0.03^{b}
1-Pentanol	0^{a}	0^{a}	0.002^{a}	0.01^{b}
1,2-Methoxypropoxy-2-propanol	0^{a}	0^{a}	0.02^{a}	0.05^{b}
Octanol	0^{a}	0^{a}	0.0026^{a}	0.01^{b}
Phenol	0^{a}	0^{a}	0.02^{a}	0.07^{b}
Propanoic acid	0^{a}	0^{a}	0.0035^{b}	0.01^{c}
Butanoic acid	0^{a}	0^{a}	0.01^{a}	0.03^{b}
Ethyl acetate	0^{a}	0^{a}	0.01^{a}	0.02^{b}
Nonanal	0.01^{ab}	0.01^{a}	0.03^{b}	0.05^{c}
Decanal	0.01^{a}	0.01^{a}	0.01^{b}	0.03^{c}
Benzaldehyde	0.01^{a}	0.01^{a}	0.02^{a}	0.03^{b}
Limonene	0.02^{ab}	0.01^{a}	0.02^{a}	0.05^{b}

Values with different letters across a row are significantly different ($p \leq 0.05$).

levels at 10 °C than at 6 °C ($p \leq 0.05$). It seems that the increase in α-longipinene in lettuce could be attributed to an increase in enzyme activity of the mevalonic pathway at the higher temperature of storage. This compound has previously been found in packaged fresh-cut lettuce at 5 °C and it has been associated with a rotten odour in lettuce (Lonchamp et al., 2009). Likewise, iso-leucine and leucine are precursors of 2-methylbutanal and 3-methylbutanal (Baldwin et al., 2000). The increase in these compounds could be attributed to deterioration of protein components of membranes by higher storage temperatures.

It is important to consider that in this study, lettuces were stored in air and the volatiles were analyzed at the end of the storage time. Air storage could allow faster deterioration of lettuce in comparison with modified atmosphere packaging due to no limitation of senescence (Kader and Saltveit, 2003b). The use of the same conditions in both replicates of the experiment does, however, make the analysis consistent. Therefore the differences observed in LOX volatiles in this study are determined by the interaction of cutting direction, storage temperature and physiological condition of lettuce. Further studies which involve the use of modified atmosphere packaging could be of interest due to the type and concentration of volatiles dependent on the produce, oxygen and carbon dioxide concentrations, time and temperature of storage (Smyth et al., 1998).

4. Conclusions

Aroma compounds and respiration rates in lettuce has been analyzed as a function of cutting direction and storage temperature. It is concluded that transverse cutting is a more severe method of preparation than longitudinal cutting, based on the increase in the levels of volatiles produced through the LOX pathway. Respiration rate was not as good an indicator of stress as cutting direction. Respiration rate was mainly affected by storage temperature.

From a practical point of view, this study provides a better understanding of the changes in aroma compounds as a part of quality changes induced by the method of preparation in lettuce. Further experiments involving sensory panels, are required to show how volatile formation by transverse and longitudinal cutting affects sensory perception of consumers.

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Paper III

Influence of packaging and storage time on aroma compounds of minimally processed lettuce

Deza-Durand, K.M., Petersen, M.A., Roepstorff, M., Poll, L.

In: Hofmann, T., Meyerhof, W., Schieberle, P. (Eds.), Advances and challenges in flavor chemistry and biology. Proceedings of the 9th Wartburg Symposium. DFA, Germany, 2011, pp. 305-309

INFLUENCE OF PACKAGING AND STORAGE TIME ON AROMA COMPOUNDS OF MINIMALLY PROCESSED LETTUCE

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Abstract

This study investigates the changes in aroma compounds of minimally processed lettuce as a function of packaging and storage time. In order to achieve this, a detailed experimental study was performed on iceberg lettuce variety *Bernardinas* which was minimally processed and packaged in two different packaging films and stored at 5 ℃ for 11 days. Changes in gas composit ion and volatile compounds were assessed. The results revealed that volatiles of minimally processed lettuce are influenced by packaging film and storage period, where the formation of off-odor limited the shelf-life of the product.

Introduction

Iceberg lettuce (*Lactuca sativa* L.) is one of the most popular vegetables used for minimally processed products. Minimally processed products are fresh vegetables, cut, washed and packaged ready-to-use [1]. Cut lettuce is sold in retail food chains, but they have a short shelf-life due to rapid loss of freshness. Browning has been pointed as the main limitation of shelf-life in cut lettuce [2]. Modified atmosphere packaging (MAP) has showed to be effective to reduce the browning and increase the shelf-life of cut lettuce using an atmosphere of 1-5% oxygen (O_2) and 5-20% carbon dioxide (CO_2) combined with low temperature of storage [3]. However, extremely low O_2 (<1%) and high CO_2 levels (>20%) can cause the production of off-odor that can be a serious limiting factor for the shelf-life of the product [4]. Therefore, the volatiles of lettuce are an important parameter to evaluate the quality of this commodity.

Key odorants reported in lettuce are cis-3-hexenol, trans-2-hexenal, trans-α-bisabolene, α-copaene, valencene, germacrene, α-terpinolene and 2-methoxy-3-isobutylpyrazine [5, 6]. However, anaerobic conditions caused by harmful MAP could decrease the key odorants of the product [7] and thus increasing the formation of volatiles which are characteristic of fermentation. Therefore, the objective of this study is to investigate the changes in aroma compounds of cut lettuce as a function of packaging and storage time.

Experimental

Iceberg lettuce variety Bernardinas was harvested in September 2009 by local grower in Bogense, Denmark. Minimally processed lettuce was packaged in two different films: OPALEN 65 AF (Film 1) and OPP/PE L 2040 AF (Film 2). All treatments were made in duplicate and stored at 5 °C for 11 days. Changes in CO_2 and O_2 in the packages were measured using a gas analyzer through the storage time. The volatiles were analyzed after 1 and 11 days of storage using dynamic headspace sampling. GC-MS was used for the separation and identification of the aroma compounds. Volatile peak area was calculated on the basis of single ions and their concentration expressed as relative area.

Results and Discussions

Atmosphere analysis

A similar content of carbon dioxide and oxygen was found between F1 (Film 1) and F2 (Film 2) after 1 day of storage with 14 % O_2 and 5% CO_2 (Figure 1). As storage time increased, the accumulation of CO_2 and depletion of O_2 increased. F1 and F2 showed injurious CO_2 and O_2 levels at 11 days of storage. CO_2 content in F1 was 27%, nearly 2 times that of the content in F2. Oxygen content was below 0.05% at the end of the storage period. Accumulation of CO_2 in F1 over the storage period was possible due to film permeability. Extremely high CO_2 levels (>20%) and low O_2 (<1%) in packaged lettuce can cause anaerobic respiration [3], as exhibited in this study after 11 days of storage.

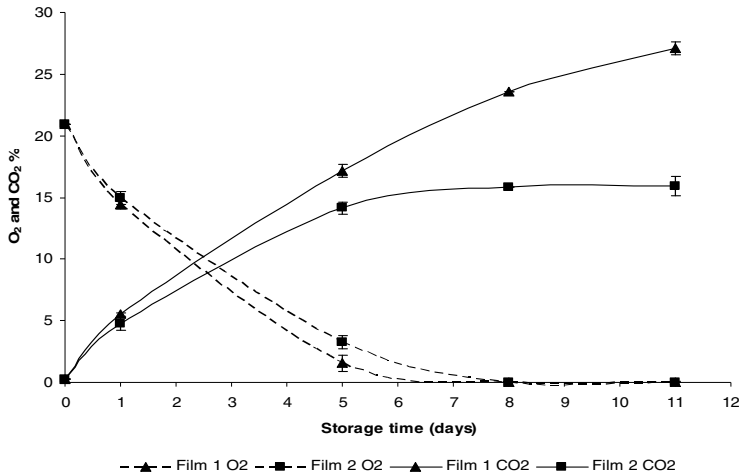

Figure 1. *Changes in O_2 and CO_2 concentration in MAP of cut iceberg lettuce stored at 5 ℃ for 11 days.*

Aroma compounds

A total of 43 aroma compounds were identified in the study. Volatiles were mainly aldehydes (14), alcohols (10), terpenes (6), ketones (5), ester (2), sulfur compounds (2), furan (2) pyrazine (1) and acid (1). Figure 2 shows the principal component

analysis (PC) on selected aroma compounds. Those volatiles were selected based on their importance as key odorants in lettuce [5, 6] and as indicators of anaerobic conditions such as 2,3-butanedione, 3-hydroxy-2-butanone, ethyl formate and ethyl acetate [8, 9].

The first principal component (PC1) clearly discriminates between storage time (66.5% of variance explained), meanwhile the second principal component (PC2) mainly separates the packaging films (24.6% of variance explained). The score and loading plots shows that lettuce packaged in F2 after 1 day of storage was related with higher amount of cis-3 hexenol, which has been indicated as key aroma in lettuce [2]. After 11 days of storage, volatiles characteristic of anaerobic respiration were observed in both film packaging. Ethyl formate, ethyl acetate, 2,3-butanedione and terpinolene were associated with F1 at 11 days of storage, whereas, trans-2-hexenal, 3-hydroxy-2-butanone, and 2-methoxy-3-isopropylpyrazine were related to F2 at 11 days of storage.

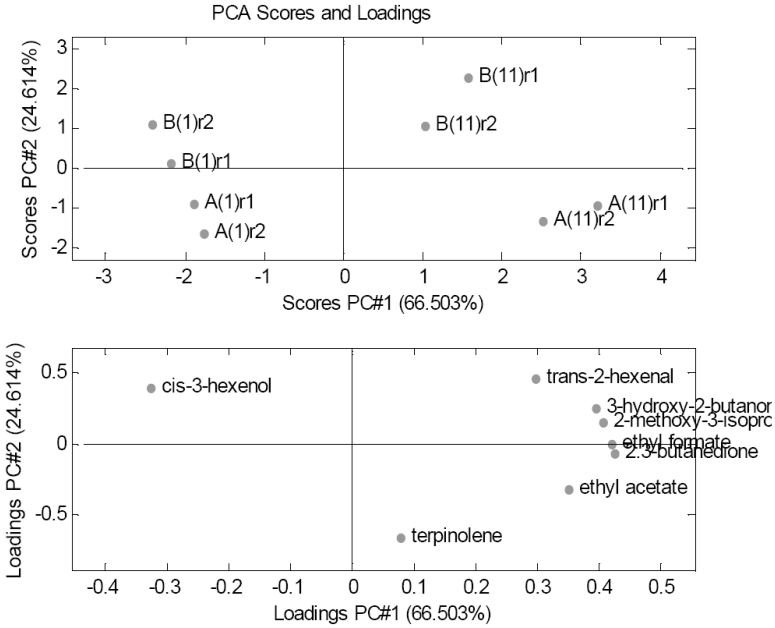

***Figure 2**. Score and loading plots of packaged cut iceberg lettuce stored at 5 ℃ after 1 and 11 days of storage. (A= Film 1, B= Film 2, 1= 1th day of storage, 11= 11th day of storage, r= replicate).*

The effect of film and storage time on the selected aroma compounds were determined by ANOVA test and the difference between means was determined by Tukey's test at 95% confidence. As observed in PC analysis, the level of cis-3-hexenol was significantly higher at day 1 of storage, meanwhile ethyl formate, 3-hydroxy-2-butanone, trans-2-hexenal and 2-methoxy-3-isopropylpyrazine increased significantly after 11 days of storage ($p \leq 0.05$) (Figure 3). An interaction was observed between packaging film and storage time, producing significantly higher levels of ethyl acetate and 2,3-butanedione after 11 days of storage in lettuce packaged in F1 than F2 ($p \leq 0.05$). Likewise, the amount of terpinolene increased significantly in F1 ($p \leq 0.05$).

This result indicates that anaerobic conditions found at the end of the storage period promote the formation of volatiles of fermentative metabolism. Anaerobic conditions could also decrease the level of key odorants, as well as, increase their concentration which may become an off-odor [7]. Off-odor was easily detected once the packages were opened at the end of the storage time, being more severe in F1 than F2 due to critical depletion of O_2 and accumulation of CO_2.

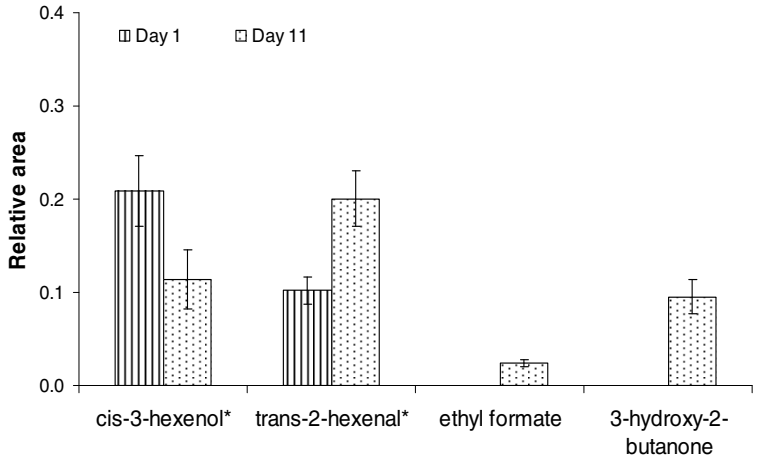

***Figure 3**. Changes of volatiles compounds in MAP of cut iceberg lettuce stored at 5℃ for 11 days. Vertical lines represented the standard error of the mean (n=4). * key odorants of lettuce.*

Conclusions

Volatiles compounds of iceberg lettuce have been analyzed as a function of packaging and storage time. Our result suggested that that packaging and storage time can have an influence on the volatiles of cut iceberg lettuce allowing the formation of desirable aroma but also on the development of off-odors that shorthen the shelf-life of the product. F1 (Film 1) is not recommended because this package developed a severe formation of off-odor at the end of the storage period due to film permeability.

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Paper IV

Effect of season, cultivar, packaging and storage time on volatile formation of cut iceberg lettuce

Deza-Durand, K.M., Petersen, M.A.

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Effect of season, cultivar, packaging and storage time on volatile formation of cut iceberg lettuce

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ABSTRACT

This study investigates the changes in volatile compounds of minimally processed iceberg lettuce as a function of season, cultivar, packaging and time. In order to achieve this, iceberg lettuce cultivars Platinas, Diamantinas and Morinas were harvested from June to September 2009. Lettuces were minimally processed and stored under three different treatments: two passive modified atmosphere packaging (MAP) built up by films of different permeabilities, F1 (OPALEN 65 AF) and F2 (OPP/PE-L 2040 AF), and storage in air. All packages were stored at 5℃. Gas composition and volatile compounds were assesse d at 1, 5, 8 and 11 days of storage in packaged lettuce, whereas in air stored samples volatiles were analyzed only at 1 and 5 days of storage. Twenty one potent odorants were identified by GC-O. Among the months, August presented a notary increase of elemene, caryophyllene, β-selinene and 2,3,-butanedione, which likely contribute to off-odour of packaged cut lettuce under anaerobic conditions. The content of O_2 and CO_2 in the treatments was demonstrated to influence the formation of odorants as storage time increased. The findings suggest that odorants arise due to different mechanisms depending on anaerobic or aerobic conditions built up during the treatments. Higher amount of cis-3-hexenol was related to aerobic conditions found in the MAP and air stored samples after 1 day of storage, whereas levels of odorants such as 2,3-butanedione, elemene, caryophyllene and β-selinene were significantly enhanced under anaerobic conditions after 11 days of storage, being

significantly higher in MAP F1. Among the cultivars, Morinas was the less tolerant to high CO_2 resulting in significantly higher amount of the undesirable odorant 2,3-butanedione.

***Keywords**: Iceberg lettuce, minimally processed, volatile compounds, gas chromatography-olfactometry, modified atmosphere packaging.*

1. Introduction

Minimally processed or cut vegetables are vegetables that have been cut in small pieces and packaged and are ready-to-eat (RTE) (Saltveit, 2003). Among them, iceberg lettuce is one of the most popular RTE vegetables in retail groceries. Over the years, the research on cut lettuce has been focused on reducing enzymatic browning by using a modified atmosphere packaging (MAP) (Heimdal et al., 1995), with little attention on the volatile formation. In Denmark passive MAP is used by the cut lettuce industry. Previous observations indicated that passive MAP successfully reduces the browning by targeting low O_2 and high CO_2 content in the packages. As a result, a product with good appearance is obtained, but the development of off-odours is a risk associated to this atmosphere, can be a serious limiting factor for the quality and marketability of cut lettuce. There is, however, relatively little information regarding volatiles of lettuce. Deza-Durand and Petersen (2011) found 45 volatile compounds in cut lettuce stored in air at 6℃. The volatiles constituted aldehydes, alcohols, terpenes, ketones, acids, sulfur compounds, acetate esters, a pyrazine and a furan. Among these compounds, copaene, 2-ethyl-1-hexanol, caryophyllene, α-longipinene, β-elemene, cis-3-hexenol, trans-2-hexenal and 2-methoxy-3-isobutylpyrazine have been identified as key odorants of cut iceberg lettuce (Arey et al., 1991; Nielsen and Poll, 2006; Lonchamp et al., 2009). Furthermore, in MAP of cut lettuce, short-chain methyl-branched alcohols and esters predominate after 10 days at 20℃ under s evere fermentation (Smyth et al., 1998).

It has been indicated that the type and concentration of volatile compounds in fruits and vegetables generally depend on cultivar, season, packaging and storage time (Smyth et al., 1998; Hodges and Toivonen, 2008). To our knowledge there is little information on volatile formation in

54 cut lettuce as function of the factors mentioned. Cut lettuce industry in Denmark uses different
55 cultivars of lettuce depending on availability during the season. This could both quantitatively and
56 qualitatively affect the formation of desirable and objectionable volatiles in the lettuce (Forney et
57 al., 2000). Likewise, season could affect the volatile formation in lettuce. For example in *Brassica*
58 specie, the changes of sulfur volatiles within a season are caused by variations in the amount of
59 aroma precursors, i.e. glucosinolates, as a result of changes in environmental conditions
60 (Mattheis and Fellman, 1999; Vallejo et al., 2003).

61 This paper aims to investigate the changes in volatile compounds of cut lettuce as a function of
62 season, cultivar, package and storage time. The outcomes of this research are relevant for the
63 cut lettuce industry as a criterion for a high quality product.

64

65 **2. Materials and methods**

66 *2.1. Plant material*

67 Three different cultivars of Iceberg lettuce (*Lactuca sativa* L. var *capitata*), Platinas, Morinas
68 and Diamantinas, were obtained from Rijk Zwaan, Odense, Denmark and sowed on 24th March,
69 11th May, 10th June, 27th June in 2009 in the commercial facilities of Bladgrønt, Denmark.
70 Cultivars were transplanted once lettuces had 4 to 6 leaves per plant to an open field in Bogense,
71 Fyn, Denmark on 20th April, 1st June, 6th July and 15th July. The harvest of lettuce was carried
72 out after 51, 49, 43 and 55 days from transplanting on 10th June, 20th July, 18th August and 8th
73 September, respectively. Lettuces were harvested based on a well formed lettuce's head and on
74 a weight range from 400 to 500 g. After harvest, maturity of lettuce was measured as firmness
75 under hand pressure (Kader et al., 1973). Lettuces were held at 1℃ for 24 h in the farm's
76 facilities and transported to a processing factory the next day.

77 *2.2. Minimally processing and packaging of lettuce*

78 Iceberg lettuces were minimally processed in the facilities of the vegetable processing factory
79 Gasa Odense, Denmark. Each lettuce cultivar was processed separately. At the factory external

leaves and core of lettuce were removed manually. Lettuces were cut in pieces around 6 mm wide with an Eillert, belt slicing machine G-1500 (Berkshire,UK). Subsequently the cut lettuce was washed in tap water at 2-5℃ for 3 min and centrifuged at 800 rpm for 10 s.

Two hundred and fifty grams of cut lettuce were weighted and packaged in two different films: film F1 (OPALEN 65 AF (65 µm)) and film F2 (OPP/PE-L 2040 AF (60 µm)). These films were commercially used by the processor. The O_2 and CO_2 transmission rate for film F1 was 35 and 158 cm^3 m^{-2} 24 h^{-1} atm^{-1} at 23 ºC and 50% RH, respectively. For film F2 the O_2 transmission rate was 68 cm^3 m^{-2} 24 h^{-1} atm^{-1} at 23 ºC and 85% RH, but no data was provided for the CO_2 transmission rate by the manufacturer Bemis Packaging (Horsens, Denmark). Cut lettuce was passive modified atmosphere packaged by the processor. Passive MAP is widely used by the cut lettuce industry in Denmark. The packages were stored overnight at the plant at 5℃ and delivered the next day to the University of Copenhagen, Department of Food Science. At the laboratory, a third treatment consisting of cut lettuce stored in air was set up. An air treatment was chosen with the finality to characterize the difference in the formation of volatiles against packaged lettuce. To this end, packaged cut lettuce of each variety were unpacked and placed in a glass jar and sealed with a household film with 40 punctured holes of 1 mm in diameter in order to maintain an atmosphere similar to air. Samples packed in F1 and F2 passive MAP were stored in duplicates for up to 11 days, whereas, air stored samples were kept for only 5 days due to browning. The storage time was calculated from the packaging of the lettuce at the plant (0 day of storage). All samples were stored at 5℃ in cool chambe rs in the laboratory (Termaks AS, Norway).

2.3. Gas analysis

CO_2 and O_2 concentrations of F1 and F2 passive MAPs were measured at 1, 5, 8 and 11 days of storage and for air stored samples at 1 and 5 days of storage. The measurements were made using a gas analyzer (Gaspace, Systech Instruments Ltd, Texas, USA) A syringe was inserted

106 inside the package to take the sample. The concentration was expressed in percentage (%) of
107 CO_2 and O_2.

108

109 *2.4. Respiration rate*

110 Respiration rate was measured in air stored samples only after 1 day of storage to obtain an
111 overview of the metabolism of lettuce at the time of packaging. To measure carbon dioxide (CO_2)
112 production, the household film on the jars was replaced with a metal lid with a septum and kept
113 for 2 h in order to obtain an accurate CO_2 measurement, above 0.5% as recommended by Kader
114 and Saltveit (2003). A syringe was inserted into the septum and the CO_2 was measured using a
115 gas analyzer (Gaspace, Systech Instruments Ltd, Texas, USA). Carbon dioxide standards were
116 used for calibration of the equipment. The respiration rate was calculated as mg CO_2 produced
117 per kg of lettuce per h on a fresh weight basis. At the end of the measurement the lid was
118 removed and the punctured household film placed again at the top of the jar.

119

120 *2.5. Dynamic headspace sampling*

121 Volatiles emitted from passive MAPs of cut lettuce were analyzed after 1, 5, 8 and 11 days of
122 storage, whereas the analysis of air stored samples was performed after 1 and 5 days of storage.
123 One hundred grams of cut lettuce was blended with 100 mL of tap water and 2 mL of internal
124 standard (50 μg mL^{-1}, 4 methyl-1-pentanol, Sigma Aldrich) was added to verify the performance
125 of the sampling. The sample was homogenized for 15 s using a blender (Struers Kebo lab) and
126 poured in a 1 L gas washing flask. The blender cup was washed with 50 mL of tap water that was
127 added to the suspension and the flask was closed with a purge head. The sample was
128 equilibrated for 10 min in a water bath of 30℃ under ma gnetic stirring (200 rpm) and then purged
129 with nitrogen (100 mL/min) for 25 min. The volatiles were trapped in a stainless steel trap
130 containing 250 mg of Tenax-TA, mesh size 60/80 and density of 0.37 g mL^{-1} (Buchem bv,
131 Apeldoorn, The Netherlands).

132

133 *2.6. Gas chromatography (GC)-Mass spectrometry (MS)*

134 The volatiles collected in the traps were thermally desorbed using an automatic thermal
135 desorption device (ATD 400, Perkin Elmer, Norwalk, USA). Tenax-TA traps were primary
136 desorbed by heating to 250℃ with helium flow of 60 mL min^{-1} for 15 min and volatiles were
137 focused in a cold trap which subsequently was flash heated to 300℃ and held for 4 min. A split
138 ratio of 1:10 was applied during transfer of the volatiles to GC-MS for their separation and
139 identification. The gas chromatograph-mass spectrophotometer used was a G1800 GCD System
140 (Hewlett-Packard, Palo Alto, CA, USA) equipped with a DB-Wax capillary column (30 m x 25 mm
141 x 0.25 µm) (J&W Scientific). The column flow rate was 1.0 mL min^{-1} using helium as a carrier gas.
142 The temperature of the column was held at 45℃ for 1 0 min and then increased by 6℃ min^{-1} up
143 to 240℃, which was kept constant for 10 min. The mas s spectrometric detector operated in
144 electron ionization mode and scanned mass/charge (m/z) ratios between 15 and 300. All volatile
145 identification was made by matching obtained mass spectra with those in the G1035A Wiley
146 library (Hewlett-Packard, Palo Alto, CA, USA) and by comparison of the retention time and mass
147 spectra with those of authentic reference standards. Volatile peak areas were calculated on the
148 basis of single ions and their concentration expressed as relative area by dividing the peak area
149 of the volatile by the area of the internal standard.

150

151 *2.7. Gas chromatography-Olfactometry (GC-O)*

152 Volatiles from lettuce samples were trapped as described above. The traps were thermally
153 desorbed using a short-path thermal system (Scientific Services Inc. NJ). The volatiles desorption
154 was done at 250℃ for 4 min with a helium flow of 10 mL min^{-1} and a split ratio 1:20. A Hewlett-
155 Packard 5890 gas chromatograph equipped with an FID detector was used. The separation of
156 volatiles was carried out using same column, carrier gas and temperature program as above. The
157 FID temperature was set at 250℃ using air and hydro gen flow of 345 mL min^{-1} and 35 mL min^{-1},
158 respectively. For the GC-O analysis, the flow was split 1:4 between the FID and an olfactory

159 detector outlet ODO-1 (SGE, Ringwood, Victoria, Australia). The flow from the outlet was mixed
160 with humidified air at 150 mL min^{-1} in order to prevent the drying of the nose mucous membrane
161 of the judges.

162

163 *2.8. Sniffing procedure*

164 GC-O was carried out in September in samples of lettuce cultivar Morinas packaged in film F2
165 at 1 and 11 days of storage. This cultivar was chosen based on its good overall quality at the field
166 and during the storage time. Ten people among the staff of the department were recruited as
167 judges for the GC-O analysis. The judges were instructed to indicate start and finish of the odour
168 (by saying 'start' and 'stop') and to describe the odour of the volatiles. During the 40 min sniffing
169 session the judge's voice was recorded digitally using a WavePad Audio Editing Software (NCH
170 software). Volatile compounds perceived by at least 3 judges were considered potent odorants.

171

172 *2.9. Statistical Analysis*

173 Multivariate analysis, partial least square discriminant analysis (PLS-DA) was made on 14
174 potent odorants detected by GC-O, and O_2 and CO_2 content, using Latentix Ver. 2.00 (Latent5,
175 Copenhagen, Denmark, www.latentix.com). PLS-DA was carried out for discrimination of potent
176 odorants among season and storage time. PLS-DA models were fully cross validated.

177 The effect of season (S), cultivar (C), packaging (P) and storage time (T) was evaluated by a
178 four-way ANOVA. The potent odorants were analyzed in two sets in order to avoid problems with
179 unbalance in the interaction P x T, because air stored samples were only analyzed at 1 and 5
180 days of storage. In the first set, the three main factors (C, S, P) with the respective levels were
181 analyzed at 1 and at 5 days of storage. In the second set, films F1 and F2 were analyzed
182 together with levels of S, C and T. The variations in O_2 and CO_2 in passive MAP samples were
183 also subjected to four-way ANOVA. Respiration rate data was analyzed by two-way ANOVA with
184 season (S) and cultivar (C) as factors.

185 Significance of difference between means for potent odorants, gas composition and respiration
186 rate were determined by Tukey's multiple range test at the 95% confidence level. Means and
187 ANOVA were performed with Infostat Statistical Software (Di Rienzo et al., 2008).

188

189 **3. Results and discussions**

190 *3.1. Respiration rate*

191 In this study, respiration rate of cut lettuce from cultivar Morinas, Platinas and Diamantinas was
192 measured in air as soon as they arrive to the laboratory facilities from the processing plant (after
193 1 day of storage). The measurements were performed in June, July, August and September 2009
194 in order to have an overview of the metabolism of lettuce (Wills et al., 1982).

195 During the season, mean value of respiration rate was around 100% higher in lettuces
196 harvested in August in comparison with June, July and September ($p \leq 0.05$) (Table 1). It is
197 important to mention that lettuces harvested in June and August were mature, whereas those
198 from July and September were over-mature, irrespective of the cultivar. Maturity is not a factor
199 under study on this research, but it is known to influence the respiration rate of vegetables and
200 fruits (Wills et al., 1982; Kays et al., 1991). Regarding the cultivars, respiration rate of cultivar
201 Morinas and Platinas were significantly higher than Diamantinas ($p \leq 0.05$), but these differences
202 seemed to be minor in comparison with season by the fact of differences were mainly around 8%.

203

204 *3.2. Gas composition*

205 As expected, the atmosphere of air stored samples (jars sealed with a perforated film) was
206 equal to air (20.9% O_2 and 0.03% CO_2). Therefore, only changes of O_2 and CO_2 in the headspace
207 of F1 and F2 passives MAPs evaluated in June, July, August and September are shown in Fig.1.
208 In both packages, the O_2 content was reduced rapidly to 0.02% after 5 day of storage in the
209 months of June, July and August ($p \leq 0.05$) while in September, the O_2 level was significantly
210 higher at the same storage time with 3.6% for F1 and 4.0% for F2 ($p \leq 0.05$), but after 8 days of

storage it was below 0.1%. The CO_2 content in packages made of film F1 was 50% higher than F2 after 11 days of storage, despite of the season. The highest level of CO_2 was reached in June with 42% and 28% for F1 and F2 at the end of the storage period ($p \leq 0.05$). The lowest CO_2 content was reached in September with 25% and 16% for F1 and F2 respectively after 11 days of storage. Irrespective of film type and season, no differences were found in O_2 and CO_2 content between cultivars, indicating only minor differences in respiration rate between cultivars.

In this study, F1 and F2 passive MAPs allowed rapid development of anaerobic conditions after 5 days and high accumulation of CO_2 at the end of the storage time, with exception of September samples. The differences in O_2 and CO_2 for both films within season could be due to differences in respiration rate caused by climatic conditions within season and/ or maturity, as explained in Section 3.1. Attention must be given to June samples, where the accumulation of CO_2 was the highest. However initial respiration rate in this month did not indicate high metabolism, thus, the increase in CO_2 might have been induced by a biological stress e.g. big vein virus. It is important to mention that in June was observed that some lettuces had big vein virus. Due to this disease, lettuces was sorted out as much as possible, however some plants could be infected without showing symptoms and might have caused the high CO_2 observed in packages samples in this month. It has been indicated that virus such as lettuce mosaic can increase the respiration rate of iceberg lettuce held in air at 2.5 ºC (Kader and Saltveit, 2003). A proper assessment of the effect of this disease on gas composition and volatile formation is, however, not within the scope of this paper.

Likewise, a higher accumulation of CO_2 in passive MAP F1 was probably due to a lower CO_2 transmission rate of film F1 in comparison with F2. It is known that a film with low CO_2 transmission rate results in a high CO_2 accumulation in the package (Jacobsson et al., 2004). CO_2 transmission rate of film F2 was not provided by the manufacturer, but the lower accumulation observed in this film supported our assumption.

238 *3.3. Volatile compounds*

239 *3.3.1. Volatile compounds and potent odorants of packaged cut lettuce*

240 Fifty two compounds were tentatively identified using GC-MS in the current study and thirty
241 one of those identifications were confirmed by running authentic standards (Table 2). The
242 volatiles of cut lettuce mainly consisted of aldehydes (13), alcohols (11), terpenes (9), ketones (5)
243 acids (4), sulfur compounds (2), acetate esters (2), furans (2), pyrazine (1) and miscellaneous (3).
244 Twenty one volatile compounds including 8 unknowns were detected by GC-O. To our
245 knowledge, 2/3-methylbutanal, 1-penten-3-ol, dimethyl sulfide (DMS) and 2,3-butanedione are
246 reported for first time as potent odorants in packaged cut lettuce (Table 3). In cut lettuce, cis-3-
247 hexenal, trans-2-hexenal and cis-3-hexenol, terpenes and 2-methoxy-3-isopropylpyrazine
248 contributed to the green leafy aroma of lettuce. It is important to note that the number of odorants
249 increased during storage time. 2,3-Butanedione, hexanal, β-selinene and seven unknowns were
250 detected only after 11 days of storage. These compounds were most probably contributors to off-
251 odour due to their sweet, unpleasant and spoiled vegetables aroma notes. Likewise, changes in
252 the concentration of desirable aroma compound can produce off-odour (Belitz et al., 2004; Poll et
253 al., 2006). For example, elemene and/or caryophyllene contributed to the leafy aroma of lettuce
254 after 1 day of storage, but were after 11 days perceived as strong chemical due to higher
255 concentration. The use of and/or indicates an uncertainty of which of both compounds was
256 responsible for the odour.

257

258 *3.3.2. Production of potent odorants of packaged cut lettuce within season*

259 A PLS-DA was done to discriminate the odorants within season. To this end a model was
260 developed using the 13 potent odorants identified by GC-O and gas composition of passive MAP
261 and air stored samples of cultivars Platinas, Morinas and Diamantinas stored for up to 11 days
262 (X) in June, July, August and September 2009 (Y) (Fig. 2).

263

From the score plot can be seen that PC1 and PC2 discriminate within season. Changes in potent odorants within season could be attributed to climatic conditions, which could explain their position along PC1. In the other hand, lettuces harvested in June and August were mature in comparison with over-mature lettuces harvested in July and September, which attributed their position along PC2. It is important to mention that the term maturity refers to commercial maturity in this study. Commercial maturity is defined as the stage of development when a plant or plant part has characteristics for an economical utilization for a particular purpose by the consumer (Shewfelt, 1986, Saltveit, 2002), thus, might be difficult to delineate changes in volatiles in comparison with physiological maturity (unknown in this study) (Wills et al., 1982; Suojala, 1999). Even thought, the authors believed that effect of physiological maturity may be irrelevant for the formation of volatiles in lettuce due to the lack of ripening phase (Wills et al., 1982). Ripening is a phase of physiological maturity in fruits in which volatiles are mainly synthetized (Wills et al., 1982). Therefore, an exhaustive study is needed in order to establish a proper relation between maturity and volatiles of cut lettuce. This is not within the scope of this paper, as such, it is suggested to perform further research on this respect.

In order to understand the formation of odorants within season, a comparison between months with lettuces with same maturity was undertaken. June and August and July and September were taken as example, as shown in the loading plot of Fig. 2. It is assumed that volatiles in lettuce are formed through metabolic routes previously identified in vegetables (Salunkhe and Do, 1976; Chin and Lindsay, 1993; Toivonen, 1997; Baldwin et al., 2000; Belitz et al., 2004; Reineccious, 2006). Thus in June cooler temperatures and long days (Table 4) may have promoted the synthesis of amino acid such as valine and s-methylmethionine for the formation of 2-methoxy-3-isopropyl pyrazine and DMS. In contrast, August was mainly characterized by having high air temperature. This condition stressed the lettuce by the fact of a high metabolism was found in August, as explained in section 3.1. This could reduce the tolerance of cut lettuce to anaerobic conditions and accelerate the deterioration, resulted in an enhancement of 2, 3 butanedione, caryophyllene, β-selinene and elemene, which are likely to be off-odours. Smyth et al. (1998) suggested that the tolerance to anaerobic conditions can change within season and other factors,

292 affecting the formation of objectionable volatiles in packaged cut lettuce. This indicates that
293 probably it would be more difficult to maintain low production of potent odorants likely to be off-
294 odours in August.

295 Moreover, when comparing over-mature lettuces of July and September, environmental
296 conditions in September e.g. less sunshine, may have reduced the amount of primary metabolites
297 amino acids and sugars for the production of most odorants. There is no information on volatile
298 biosynthesis in lettuce regarding the influence of the environment, but in strawberries it has been
299 seen that a short period of low light by shading reduced photosynthesis, resulting in less primary
300 metabolic products for volatile formation (Watson et al., 2002). Likewise, in September aerobic
301 conditions were observed for longer period in passive MAPs (around 4% after 5 days of storage).
302 This result could indicate that most odorants probably were enhanced by anaerobic conditions
303 due to membrane deterioration. Therefore, it seems unlikely that off-odours were a problem in
304 September, but other problems might arise, since it has been indicated that over-mature lettuces
305 are more prone to postharvest problems (Cantwell and Suslow, 2002).

306

307 *3.3.3. Production of potent odorants of packaged lettuce as storage time increase*

308 A PLS-DA model was made on 13 odorants and gas compostion of data from passive MAPs
309 and air stored samples of cultivars Platinas, Morinas and Diamantinas harvested in June, July,
310 August and September 2009 (X) stored for up to 11 days (Y). From the score plot, it can be seen
311 that all the samples were displaced clockwise from day 1 to day 11 of storage. The displacement
312 of samples evidenced changes in the amount of potent odorants as storage time increased.

313 After 1 day of storage, aerobic conditions predominated in the passive MAPs and air stored
314 samples, which seemed to increase the level of cis-3-hexenol. As storage time increased the
315 level of O_2 decreased and the CO_2 content increased in the passive MAPs. Therefore, most of the
316 odorants seemed to increase after 8 days of storage, after being exposed to extremely low O_2
317 and high CO_2. Further exposition, up to 11 days, enhanced the formation of odorants that were
318 described as unpleaseant such as 2,3-butanedione, β-selinene, caryophyllene and elemene. It is

319 believed that 2,3-butanedione could also be produced by microorganism under anaerobic
320 conditions (Syu, 2001). However, microbial growth and volatiles formation by microorganism was
321 not included in this study. Further studies are suggested to investigate the influence of microbial
322 growth in the formation of volatiles in cut lettuce.

323 It is clear that changes in the formation of odorants as storage time increased could be the
324 result of membrane deterioration by prolonged exposition to extremely low O_2 and high CO_2. It
325 has been reported that cellular deterioration enhanced the formation of volatiles in cabbage (Chin
326 and Lindsay, 1993) and broccoli (Di Pentima et al., 1995; Jacobsson et al., 2004). Potent
327 odorants at higher concentration can become off-odours (Poll et al., 2006). Therefore, MAP of cut
328 lettuce cannot be stored for up to 11 days due to the increase of odorants likely to be off-odours.
329 After 11 days of storage off-odour was easily detected once the packages were opened, being
330 more severe in passive MAP F1. The off-odour was described as rotten, although no formal
331 sensory analysis was performed. In terms of odor, probably, cut lettuce packaged in MAP could
332 be stored for up to 8 days. This point of time seemed to be where the early stage of enhancing
333 odorants by anaerobic conditions started. Proper sensory studies are necessary to determine the
334 presence of off-odours at this storage time.

335

336 *3.3.5. Production of odorants as function of season, cultivar, packaging and storage time.*

337 Finally a four-way ANOVA was performed in order to determine if the differences observed in
338 the multivariate analysis for some odorants were significant. It is noteworthy to mention that PLS-
339 DA was also carried out between odorants and cultivars. However, no good correlation and
340 separation was achieved and for that reason were not presented in this study. The analysis was
341 focused on elemene, caryophyllene, β-selinene, DMS and 2,3-butanedione that are likely to be
342 off-odours under anaerobic conditions. LOX volatiles such as cis-3-hexenol, trans-2-hexenal and
343 hexanal were also selected since they are important indicators of stress (Deza-Durand and
344 Petersen, 2011; Hildebrandt, 1998).

345 Fig. 4. shows the changes in levels of some selected odorants during storage. Cis-3-hexenol
346 decreased significantly after 5 days of storage in packaged and air stored samples ($p \leq 0.05$), with
347 no differences between them. As storage time increased, the drop of cis-3-hexenol increased in
348 magnitude, being significantly lower after 11 days of storage in passive MAP F1 ($p \leq 0.05$). On the
349 contrary, hexanal increased over the storage time in passive MAPs samples, being significantly
350 higher after 11 days of storage in passive MAP F1 ($p \leq 0.05$). No differences were found in
351 hexanal for air stored samples between 1 and 5 days of storage. Among terpenes, elemene was
352 higher in passive MAP F1 after 11 days of storage (Fig 4), whereas the relative area of
353 caryophyllene and β-selinene significantly increased in June and August after 11 days of storage
354 in passive MAP F1, mainly for cultivar Diamantinas for β-selinene ($p \leq 0.05$). Furthermore,
355 terpenes were sharply reduced after 5 days of storage in air stored samples ($p \leq 0.05$). Moreover,
356 the amount of DMS in both passive MAPs increased significantly after 5 days of storage in June
357 and August than in July and September ($p \leq 0.05$). However, after 11 days of storage the level of
358 DMS decreased significantly ($p \leq 0.05$). For air stored samples, the amount of DMS tended to
359 increase, but it was significantly lower than passive MAPs in June ($p \leq 0.05$).

360 Among cultivars, differences in the formation of individual potent odorants were not significant,
361 with exception of 2,3-butanedione which was significantly higher in the cultivars Morinas and
362 Diamantinas after 11 days of storage in passive MAP F1 in August ($p \leq 0.05$) (Fig. 5). It is
363 important to mention that this volatile was not found in air stored samples due to its anaerobic
364 nature.

365 These results support earlier observations from PLS-DA analysis. In both passive MAPs,
366 probably membrane deterioration due to extremely low O_2 and high CO_2 was the main cause for
367 the increase of odorants, mainly in passive MAP F1 after 11 days of storage. Among the cultivars,
368 Morinas might be less tolerant to extreme atmosphere by the fact of a high production of 2,3-
369 butanedione, which is a volatile characteristic of anaerobic conditions. This tolerance to extreme
370 atmosphere seemed to be affected by season since it was mainly observed in August, as
371 explained in Section 3.3.2. It is noteworthy, that off-odour was not detected in air stored samples
372 after 5 days of storage, although trans-2-hexenal and DMS increased. Absolute values of

373 volatiles in this study were not calculated, thus it made difficult to state that were high enough to
374 be detected in the headspace of the jars.

375 Moreover, we cannot exclude the effect of low O_2 and high CO_2 in the mechanism of formation
376 of volatiles. Thus, compounds might have arisen from different mechanisms under aerobic and
377 anaerobic conditions. For example in LOX volatiles, there was an enhancement in the production
378 of trans-2-hexenal over the formation of cis-3-hexenol in the presence of air after 5 days of
379 storage. The formation of these compounds could be partially due to enzymatic degradation
380 through the LOX pathway and autoxidation (Galliard et al., 1977; Riley et al., 1996). Moreover,
381 the formation of aldehyde over alcohol could be part of the senescence process of the lettuce
382 under air conditions and under low O_2 (Belitz et al., 2004). However, the increase in hexanal
383 could be a result of the formation of hydroperoxides during cutting by LOX enzyme (before
384 packaging). After packaging, hydroperoxides could be degraded to aldehydes by hydroperoxide
385 lyase enzyme (Belitz et al., 2004), in a process that does not require O_2.

386 Sesquiterpenes such as elemene and caryophyllene are produced by the mevalonate pathway
387 (MVA) in the cytosol (Tholl, 2006). Their decrease in air stored samples could be attributed to
388 oxidation process during storage (Belitz et al., 2004). However, under anaerobic conditions, the
389 MVA route could be diminished due to acetyl CoA is no longer produced to initiate the route
390 (Belitz et al., 2004; Tholl, 2006). Therefore, an alternative route, the methylerythritol phosphate
391 (MEP) pathway could be enhanced due to accumulation of pyruvate, which reacts with D-
392 glycerol-3-phosphate to initiate this route (Belitz et al., 2004; Tholl, 2006) by the fact that pyruvate
393 is accumulated under anaerobic conditions (Siriphanich and Kader, 1986). Recent studies in
394 carrots by Hampel et al. (2004) demonstrate that sesquiterpenes, could be produced either by
395 MVA and MPE route, where isopentenyl diphosphate and/or dimethylallyl diphosphate produced
396 by MEP pathway were transported from plastids to cytosol for the formation of sesquiterpenes. It
397 is assumed that similar behavior might have occurred in lettuce.

398 S-methylmethionine sulfonium salt has been indicated to be a precursor of DMS in broccoli
399 (Jacobsson et al., 2004). Di Pentima et al. (1995) and Dan et al. (1997) indicated that extremely

low O_2 and high CO_2 caused membrane deterioration in broccoli which enhanced the availability of S-methylmethionine sulfonium salt for the formation of DMS. Membrane deterioration by extremely low O_2 and high CO_2 could explain the increase in DMS in packaged lettuce. Likewise, the reduction of DMS after 11 days of storage probably was caused by a drop of the enzyme activity that yield this volatile due to a decrease in the pH of lettuce and acidification. Lettuce pH was near 4.5 in packaged samples after 11 days of storage as consequence of high CO_2 (Siripanich and Kader, 1986) (data not shown). Lewis et al. (1971) and Chin and Lindsay (1993) found that a deficiency of DMS in cabbage homogenates could be attributed to reduction of the enzymatic reaction on S-methylmethionine sulfonium salt under low pH.

4. Conclusions

In this study, 52 volatile compounds were identified and of these 21 were shown to contribute to the aroma of packaged cut lettuce. Among them elemene, caryophyllene, β-selinene and 2,3-butanedione, enhanced under anaerobic conditions and likely to be off-odours. In August high production of these odorants was found and probably compromised the quality in terms of odour. Both passive MAPs, F1 and F2, developed extremely low O_2 and high CO_2 content, which enhanced the formation of odorants likely to be off-odours and the loss of others such as cis-3-hexenol through the storage time. The passive MAP built with film F2 (relatively high carbon dioxide permeability) seemed to be the most promising packaging because it kept the concentration of elemene, caryophyllene, β-selinene and 2,3-butanedione lower than film F1. However, proper sensory evaluations are needed in order to confirm this conclusion. Regarding the cultivars, Morinas and Diamantinas increased the amount of objectionable odorants such as 2, 3-butanedione under anaerobic conditions.

This study concludes that the presence of off-odour can be a limiting factor for shelf-life of packaged cut lettuce. In order to minimize their formation, cultivar Platinas should be preferred over Morinas and Diamantinas, and film F2 probably should be preferred over F1. Storage of packages for up to 11 days should be avoided due to the increase of volatiles likely to be off-odours.

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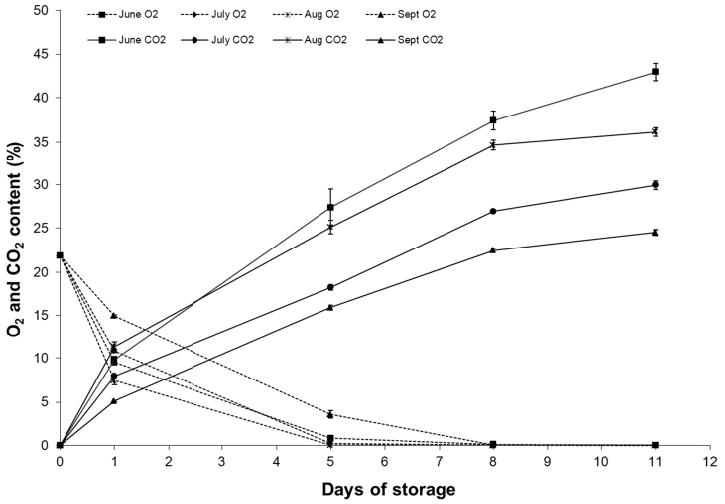
a) Passive MAP F1



b) Passive MAP F2

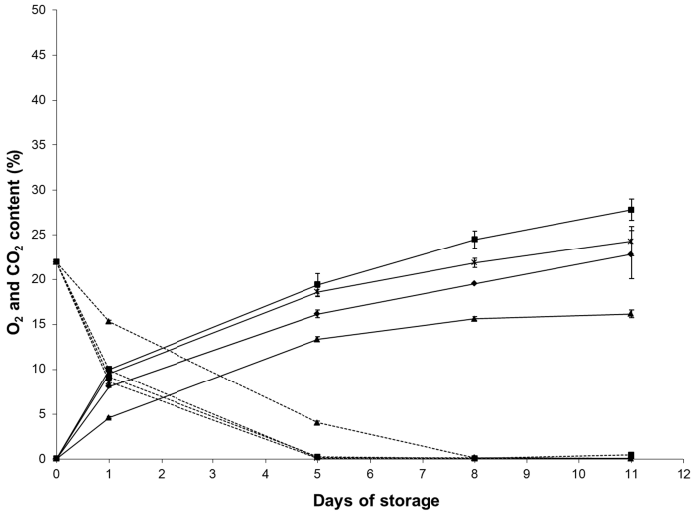


Fig. 1. Changes in O_2 and CO_2 concentration of packaged cut lettuce stored for 11 days at 5℃ in June, July, August and September 2009. The symbols are the average of the replicates of the three varieties and the vertical lines represent the standard deviation. *Abbreviations: F1,film 1; F2, film 2; Aug= August; Sept, September.*

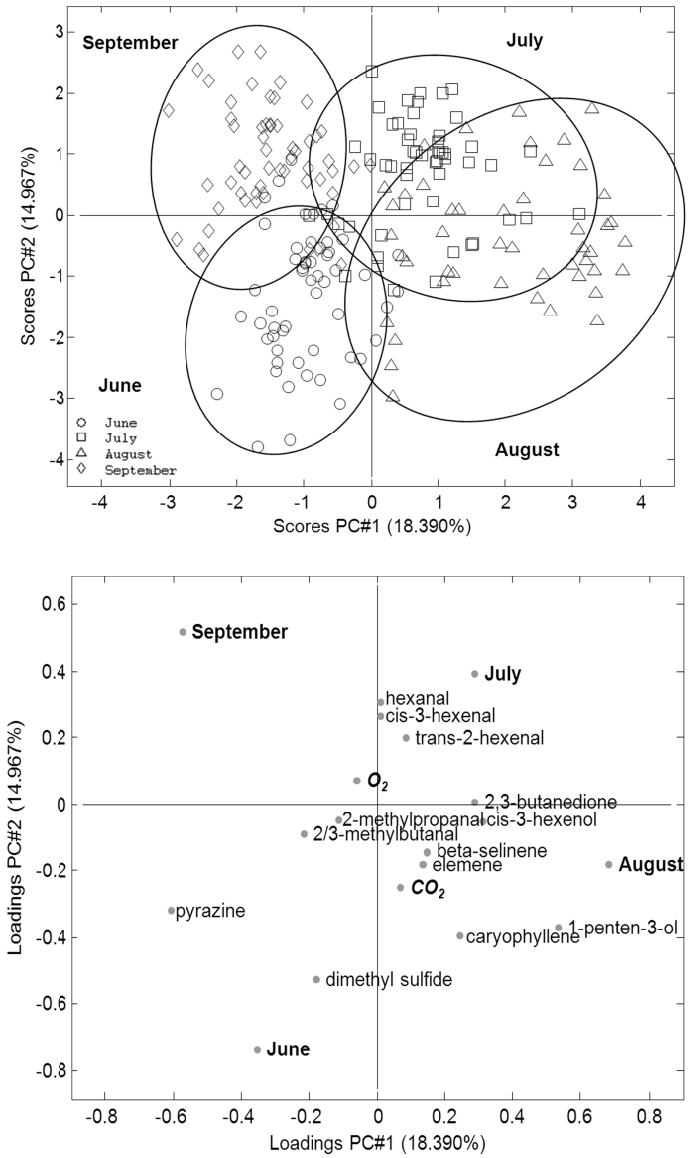


Fig. 2. A PLS-DA score and loading plots of potent odorants and gas composition from passive MAP and air stored samples of cultivars Platinas, Morinas and Diamantinas stored for up to 11 days at 5 ºC in June, July, August and September 2009.

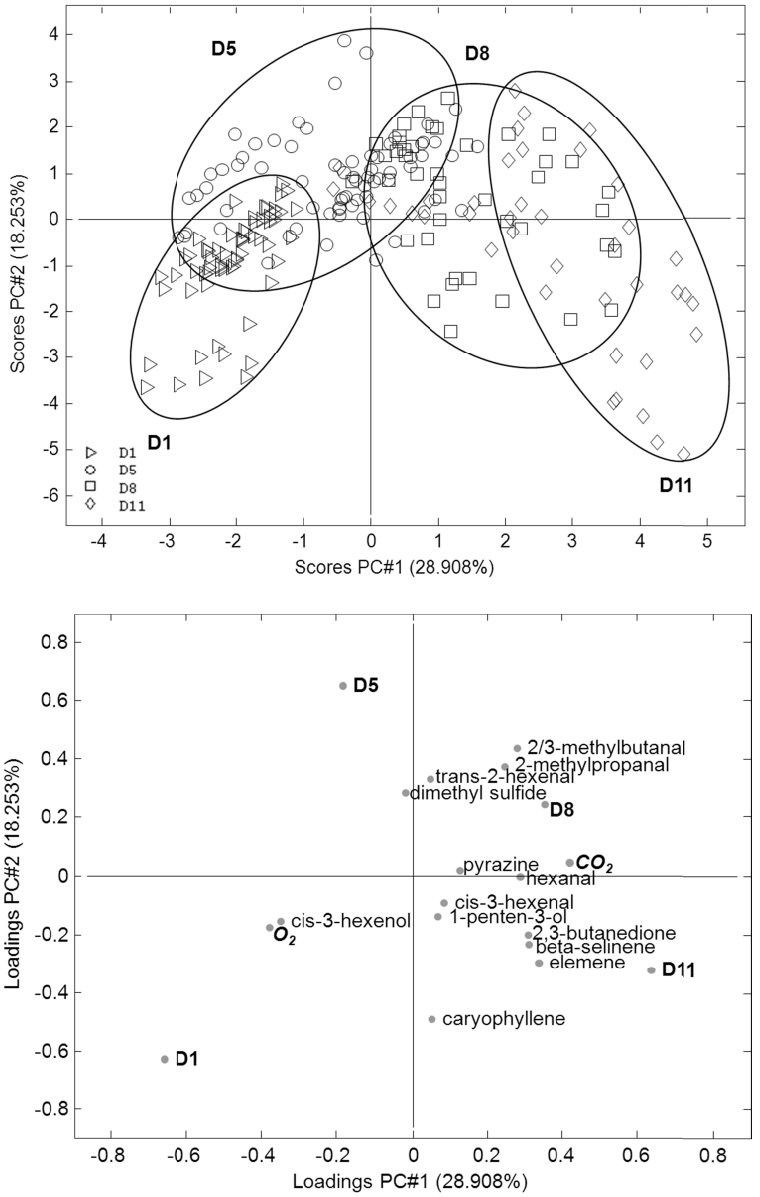


Fig. 3. PLS-DA score and loading plots of odorants and gas composition of data from passive MAPs and air stored samples of cultivars Platinas, Morinas and Diamantinas harvested in June, July, August and September 2009 stored for up to 11 days at 5℃. *Abbreviations: D1, first day of storage; D5, fifth day of storage; D8, eighth day of storage; D11, eleventh day of storage*.

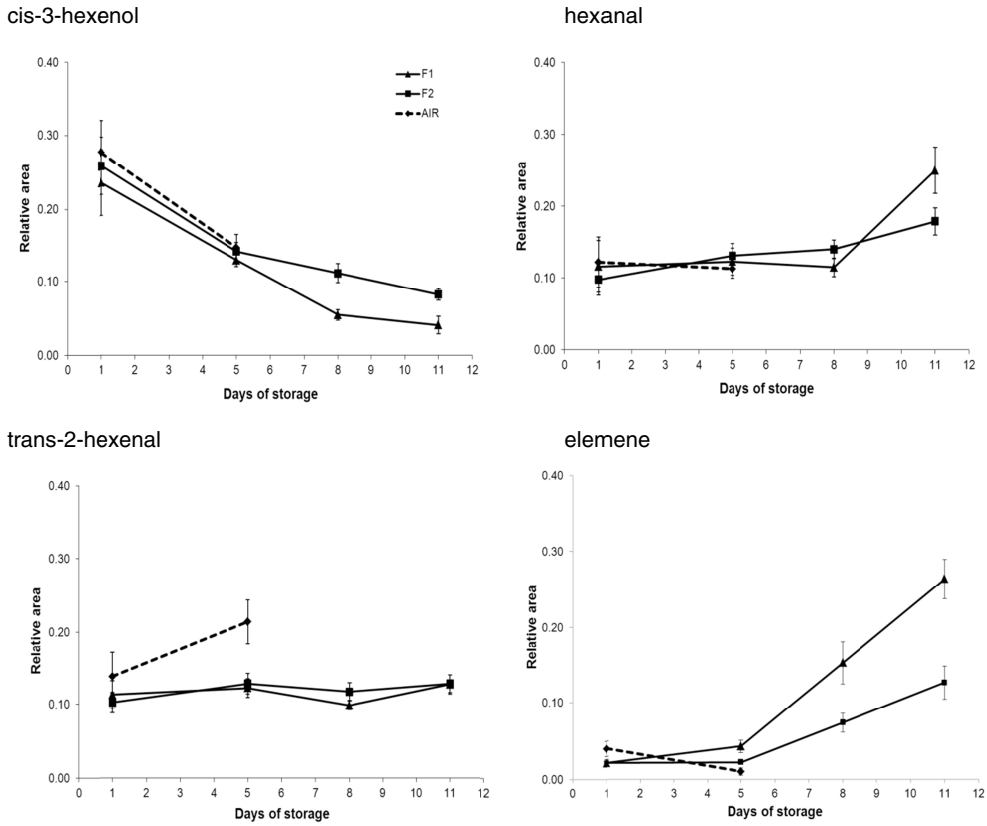


Fig. 4. Changes in the relative area of selected odorants of cut lettuce stored at 5℃ for 5 days in air and for up to 11 days in passive MAPs F1 and F2. Vertical lines represent the standard error of the mean (n=24).

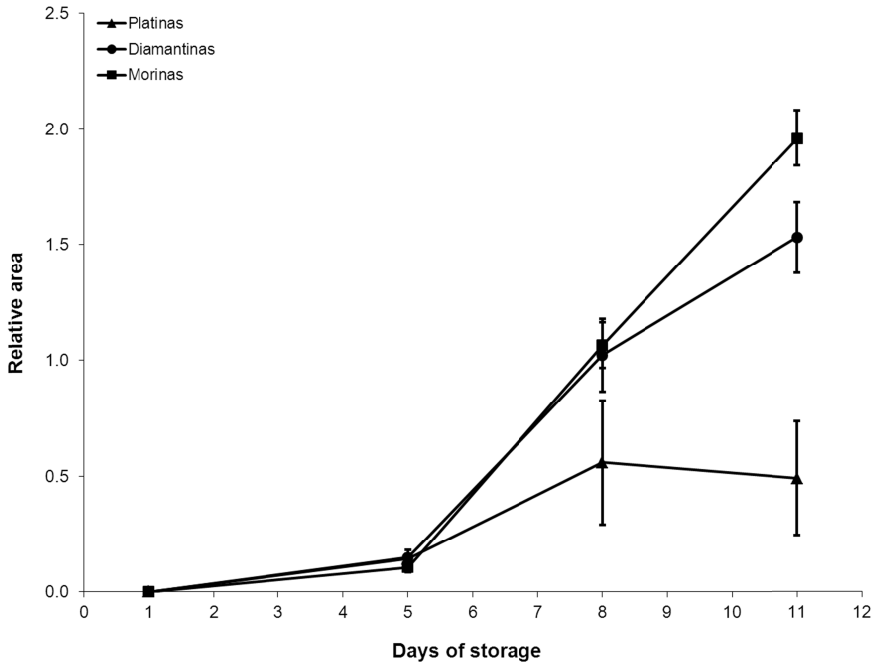


Fig. 5. Changes of 2,3-butanedione in cultivar Morinas, Diamantinas and Platinas in passive MAP F1 stored for up to 11 days in August 2009.

Table 1. Respiration rate of cultivars Platinas, Morinas and Diamantinas stored in air at 5 ℃ after 1 day of storage in June, July, August and September 2009.

Factors	Respiration rate ($mgCO_2\ kg^{-1}\ h^{-1}$)
Season	
June	59.3 ± 7.8 (9)[a]
July	60.2 ± 6.7 (9)[a]
August	112.5 ± 13.2 (9)[b]
September	60.3 ± 9.7 (9)[a]
Cultivars	
Platinas	76.7 ± 28.3 (12)[b]
Morinas	74.2 ± 28.1 (12)[b]
Diamantinas	68.3 ± 18.5 (12)[a]

Data expressed as mean ± standard deviation. Values on parenthesis represent the number of samples used for the calculation of the mean. Different letters indicate significantly differences at $p \leq 0.05$.

Table 2. Volatile compounds identified in cut lettuce stored in air and packaged in film F1 and F2 for up to 11 days at 5℃ in June, July, August and September 2 009.

Volatile compounds	
Aldehyde	*p*-cymene
2-methylpropanal	terpinolene
2/3-methylbutanal*	α-humulene
hexanal*	α-selinene
cis-3-hexenal	β-selinene
trans-2-hexenal*	***Ketone***
propanal*	2,3-butanedione*▲
2-propenal*	3-hydroxy-2-butanone▲
pentanal*	2-butanone*
octanal*	6-methyl-5-hepten-2-one*
nonanal*	acetophenone
2,4-hexadienal	***Acids***
decanal*	propanoic acid*
benzaldehyde*	butanoic acid*
Alcohol	2-methyl butyric acid*
1-butanol*	hexanoic acid*
1-penten-3-ol*	***Sulfur compounds***
cis-3-hexenol	dimethyl sulfide*
1-propanol	dimethyl sulfoxide
2-methyl-1-propanol	benzothiazole*
1-pentanol*	***Ester***
1-hexanol*	ethyl formate▲
trans-2-hexenol*	ethyl acetate*
2-ethyl-1-hexanol	***Pyrazine***
octanol*	2-methoxy-3-isopropyl pyrazines
phenol	***Furan***
Terpene	2-ethylfuran*
caryophyllene*	2-pentylfuran*
elemene	***Miscellaneous***
α-pinene	ethyl tiglate▲
limonene	styrene*

Compounds were tentatively identified based on probability based matching with mass spectra in the Wiley database. For the compounds marked with an asterisk the identity was confirmed by running authentic standards. Compounds marked with a triangle were not found in air stored samples.

Table 3. Potent odorants detected by GC-O of cut lettuce packaged in film F2 after 1 and 11 days of storage at 5℃ in September 2009.

Volatile compounds	Odour descriptor	
	1 day of storage	11 days of storage
Aldehydes		sweet
2-methylpropanal		
2/3-methylbutanal	sweet, cocoa	strong sweet, cocoa
hexanal		lettuce, fruity
cis-3-hexenal	lettuce	weak grass
trans-2-hexenal	vegetables	unpleasant, fatty
Alcohol		
1-penten-3-ol	flower	
cis-3-hexenol	alcohol, chili, soil	soil, weak grass, tea
Terpene		
elemene and/or caryophyllene	lettuce, grass, flower, soil	strong chemical, grass, chili
β-selinene		spoiled vegetables, flowers
Sulfur compounds		
dimethyl sulfide	boiled broccoli, shellfish	off-odour, broccoli, shellfish
Ketone		
2,3-butanedione		sweet, caramel
Pyrazine		
2-methoxy-3-isopropylpyrazine	lettuce, soil, mushroom	grass, soil
Others		
Unknown 1		off- odour, curry
Unknown 2		spoiled vegetables
Unknown 3	chamomile, mint	
Unknown 4		fruity, green, pea
Unknown 5		fatty, toasted almonds
Unknown 6		mint, slightly cheese
Unknown 7		tea, chili
Unknown 8		unpleasant, cassava flour

The use of and/or indicate an uncertainty of which of both compounds was responsible for the odour.

Table 4. Weather conditions during the growing period of lettuce in an open field from April to September 2009.

	Monthly average		
	Temperature (℃)	Precipitation (mm)	Hours of sunsh ine
June	13.5	49.4	271.6
July	17.5	59.9	242.7
August	17.6	37.3	217.1
September	14.1	33.0	160.6

Data provided by the Danish Meteorological Institute at Odense Airport and weather station in Årslev.

Paper V

Changes in physicochemical characteristics of packaged and air stored cut iceberg lettuce upon storage and season

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Changes in physicochemical characteristics of packaged and air stored cut iceberg lettuce upon storage and season

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Abstract: This study investigates the changes in physicochemical characteristics of minimally processed iceberg lettuce as a function of season, cultivar, packaging and storage time. Iceberg lettuce cultivars Platinas and Morinas were harvested from June to September 2009. Lettuces were cut and packaged in two passive modified atmospheres built up using films F1 (OPALEN 65 AF) and F2 (OPP/PE-L 2040 AF) of different O_2 and CO_2 transmission rate, and storage in air. All packages were stored at 5°C. Changes in gas composition, glucose, fructose, sucrose, malic acid, tartaric acid, ascorbic acid, chlorogenic acid, firmness, browning and polyphenol oxidase activity were assessed at 1, 5, 8 and 11 days of storage in packaged cut lettuce, whereas in air stored samples the analysis was carried out at 1 and 5 days of storage. For the analysis of glucose, fructose, sucrose, malic acid, tartaric acid, ascorbic acid and chlorogenic acid a gas chromatography mass spectrometry (GC-MS) method was developed. Our results indicated that in June soluble sugars, malic acid and firmness were kept high irrespective of the package. Browning area was high in air stored samples in August and September, mainly in cultivar Platinas. Browning was remarkably controlled in packaged samples, irrespective of season and cultivar due to extremely low O_2 and high CO_2 conditions; however, after 11 days of storage this conditions favored tissue softening, decreased of sugars and malolactic fermentation, mainly in passive MAP F1.

Keywords: Iceberg lettuce; browning, image analysis, soluble sugars; organic acids; ascorbic acid; chlorogenic acid; gas chromatography mass spectrometry.

1. Introduction

Iceberg lettuce (*Lactuca sativa.* L) is one of the most popular minimally processed vegetables in retail groceries [1, 2]. Minimally processed or cut vegetables are vegetables that have been cut in small pieces and are ready-to-eat [3]. Browning has been indicated as the main limitation of cut lettuce's shelf-life [4]. Modified atmosphere packaging (MAP) has been successfully used for the reduction of browning in cut lettuce [4, 5]. The recommendations for adequate MAP for cut lettuce include ranges of 1-5% O_2 and 5-20% CO_2 at storage between 0 and 5 ºC [6]. Some investigations indicate that the lower limit of O_2 could be further reduced to between 0.3-0.5% O_2 at 5 ºC with no browning and a minimum of undesirable odors during storage for up to 6 days [5]. Lettuce from different growing season, cultivars or different agricultural practices can, however, respond differently to low O_2 and high CO_2 storage conditions [3]. In Denmark, lettuces are harvested from June to September. It is known that changes in climatic conditions during a season can influence the chemical composition in vegetables [7, 8]. For instance, in carrots an increase in the content of soluble sugars has been related to low temperate during growing [9, 10]. Most of the studies on physicochemical characteristics in cut lettuce have been focused on the effect of packaging and cultivar mainly on browning [4, 5]. To our knowledge, there is not an integrate study taking into account the influence of season, cultivar, packaging and storage time on changes in soluble sugars, organic acids, chlorogenic acid, firmness and browning.

Several methods have been developed for the analysis of soluble sugars, malic acid, tartaric acid and chlorogenic acid in lettuce by mainly using high performance liquid chromatography (HPLC) and by indophenol titration for ascorbic acid determination [4, 11, 12]. However, there has not been previous analysis of the metabolites mentioned above by gas chromatography mass spectrometry (GC-MS) in lettuce. The advantages of GC-MS rely on a simultaneous analysis of these metabolites in one chromatographic run, better separation of compounds in gas phase than in liquid phase, high sensitivity that decreases the amount of the biological material for accurate measurements and better identification power by MS due to extensive compounds database [13].

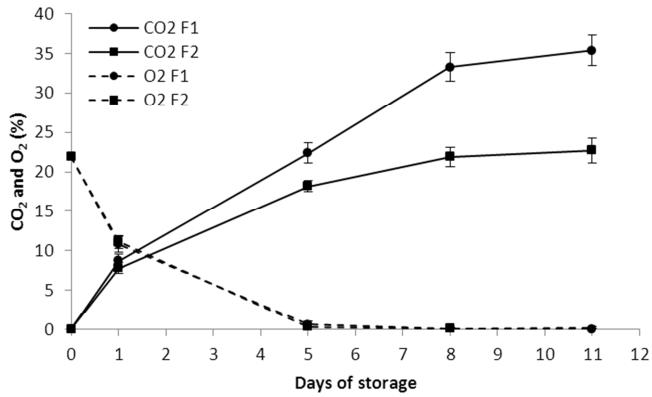
The objective of this study was to assess the influence of season, cultivar, packaging and storage time on physicochemical characteristics of cut iceberg lettuce. In the present research lettuces were cultivated by local growers and minimally processed under industrial practices which provided more realistic information about the quality of this product. This knowledge will benefit the growers and industry to improve the production process of lettuce from the farm to table.

2. Results and Discussions

2.1. Gas composition

As expected, the atmosphere of air stored samples (jars sealed with a perforated film) was equal to air (20.9% O_2 and 0.03% CO_2). Therefore, only changes of O_2 and CO_2 in the headspace of passive MAPs F1 and F2 were subjected to analysis. Both passive MAPs allowed development of anaerobic conditions after 5 days of storage (0.3% O_2) ($p \leq 0.05$). Further storage allowed the decrease of O_2 to 0.1%. Significantly higher accumulation of CO_2 was observed in passive MAP F1 than MAP F2. After 8 days of storage the content of CO_2 reached equilibrium with 33.3 and 21.9% for MAP F1 and F2 respectively ($p \leq 0.05$) (Fig. 1). This was probably due to a lower CO_2 transmission rate of film F1 in comparison with F2 [14]. It is known that a film with low CO_2 transmission rate results in a high CO_2 accumulation in the package [15]. No significant differences were found between 8 and 11 days of storage for the respective films.

Figure 1. Changes in the gas composition of the headspaces of passive MAP F1 and F2 stored at 5°C for up to 11 days. Vertical lines represent the standard error of the mean (n=14).



Irrespective of the package, the highest mean of CO_2 content was found in June with 25.3%, whereas the lowest was observed in September with 13.5% (*$p \leq 0.05$)* (Table1). In addition, a significantly higher mean of O_2 was found in September than the rest of the months (*$p \leq 0.05$)* (Table1). Differences in O_2 and CO_2 within season could be due to differences in respiration rate of lettuces caused by climatic conditions within season and/or maturity or as a result of biological stress e.g. big vein virus, as probably occurred in June [14]. Contrary to what was expected, there were not significant differences in O_2 and CO_2 content in the packages due to cultivars.

Table 1. Mean of O_2 and CO_2 content of the headspace of passive MAPs within season

	June	July	August	September
O_2 (%)	2.58 ±4.03 (31) [b]	1.78 ±4.09 (23) [a]	1.85 ±2.80 (25) [a]	6.31 ±7.69 (17) [c]
CO_2 (%)	25.26 ± 11.70 (31) [d]	20.10 ±8.34 (23) [b]	22.30 ±8.16 (25) [c]	13.54 ±8.10 (17) [a]

Data expressed as mean of film F1 and F2 ± standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters across a row indicate significant differences at $p \leq 0.05$.

2.2. Influence of season on physicochemical characteristics of packaged and air stored cut iceberg lettuce

A PLS-DA was carried out in order to discriminate the physicochemical characteristics in packaged an air stored cut lettuce within season (Fig.2). The score plot shows that only June and August were clearly discriminated. Samples of September totally overlapped with July indicating small differences in physicochemical characteristics. From the loading plot it can be seen that firmness, pH, glucose and fructose tended to had high values in June, while tartaric acid was highest in August. Most of the physicochemical characteristics tended to have low levels in July and September.

Our results show a significant main effect of season in the variation of physicochemical characteristics (fructose, glucose, sucrose, malic acid, tartaric acid, pH and firmness) of packaged cut lettuce (Table 2). Fructose, glucose and sucrose were found to be the main soluble sugars in lettuce [4, 16]. Irrespective of the package, the concentration of fructose, glucose and sucrose was significantly higher in June than the rest of months ($p \leq 0.05$). Among the sugars, the concentration of fructose was around 2 times higher than glucose and 70 times higher than sucrose. Previous studies by Heimdal *et al.* [4] and Poulsen *et al.* [16] also indicated that fructose is the main sugar in lettuce and sucrose is presented in low concentration. Moreover, we found that malic acid was the major organic acid followed by tartaric acid. The concentration of malic acid was significantly higher in June than July and August ($p \leq 0.05$). On the contrary, the lowest concentration of tartaric acid was found in June and the highest in August ($p \leq 0.05$). Firmness is an important quality characteristic of lettuce, and was significantly higher in June ($p \leq 0.05$). For pH, there was a significant interaction between season and packaging. The lowest value of pH was found in August in passive MAP F1 with a value of 5.37 ($p \leq 0.05$). Moreover, there were no significant differences between air stored samples and packaged samples (both stored for 5 days) within season ($p \geq 0.05$). Nevertheless, cut lettuce stored in air showed trends similar as to packaged cut lettuces, as explained above.

Figure 2. PLS-DA model of physicochemical characteristics and gas composition of packaged and air stored cut iceberg lettuce stored at 5 ºC in June, July, August and September 2009

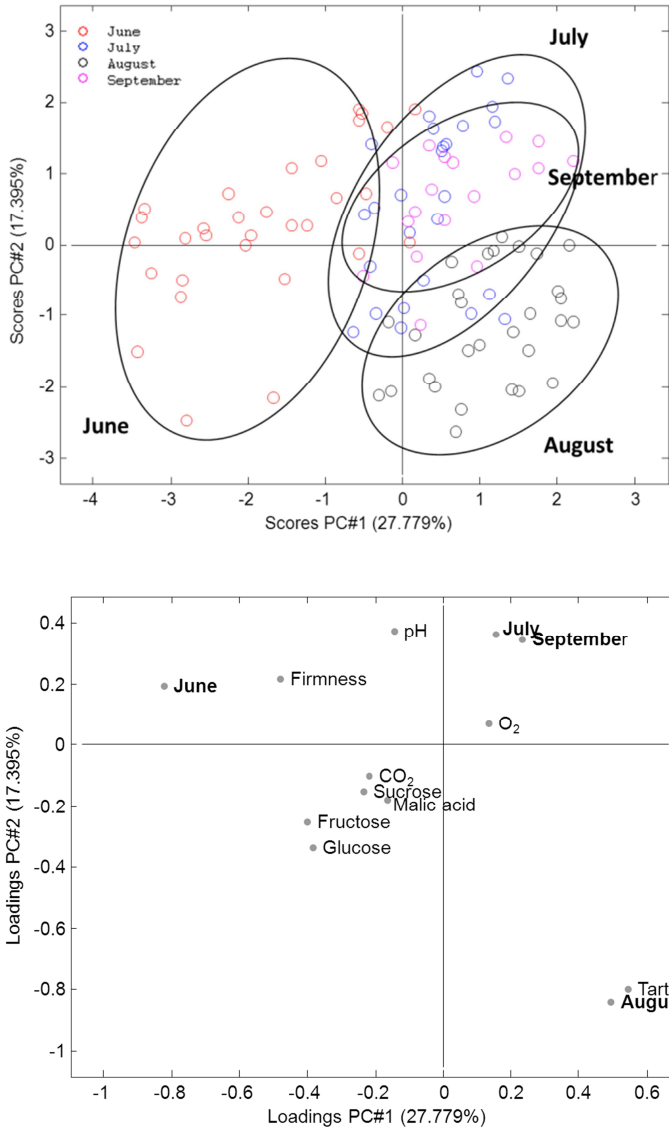


Table 2. Concentration of soluble sugars, malic acid, tartaric acid and firmness found in packaged cut lettuce stored at 5 ºC within season.

	June	July	August	September
Fructose (g/100g FW)	0.71± 0.32 (31) [c]	0.48 ±0.13 (26) [ab]	0.49 ±0.16 (26) [b]	0.36 ±0.13 (26) [a]
Glucose (g/100g FW)	0.39 ±0.20 (31) [c]	0.22 ±0.07 (26) [ab]	0.26 ±0.11 (26) [b]	0.17 ±0.09 (24) [a]
Sucrose (g/100g FW)	0.011 ±0.008 (30) [b]	0.014 ±0.007 (23) [b]	0.006 ±0.006 (25) [a]	0.005 ±0.005 (20) [a]
Malic acid (mg/100g FW)	26.50 ±17.25 (31) [b]	13.88 ±9.43 (26) [a]	16.50 ±12.90 (26) [a]	23.64 ±14.55 (24) [ab]
Tartaric acid (mg/100g FW)	1.22 ±1.18 (25) [a]	11.10 ±6.15 (23) [c]	13.65 ±5.81 (20) [d]	7.45 ±3.66 (20) [b]
Firmness (N/g)	18.82 ±1.97 (30) [c]	17.16 ±1.71 (25) [b]	15.57 ±1.57 (27) [a]	16.13 ±1.14 (24) [a]

Data expressed as mean± standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters across a row indicate significant differences at $p \leq 0.05$. Abbreviations: FW= fresh weight.

To our knowledge there is no information regarding the effect of climatic conditions on physicochemical characteristics in lettuce. Within season 2009, June was characterized by a low average temperature of 13.5 ºC and many hours of sunshine (271 hours) (Danish Meteorological Institute). In grape berries, it has been indicated that long period of photosynthetic activity results in increased production of photo assimilates such as carbohydrates [17] and malic acid [18]. In carrots, an increase in the concentration of soluble sugars was related to low temperatures during growing [9, 10]. If these effects are general, more hours of sunshine in combination with low temperatures would explain the increase of soluble sugars and malic acid in cut lettuce packaged in June. The accumulation of carbohydrates could also contribute to changes in cell wall components, which are major contributors to firmness in lettuce [19, 20]. Moreover, the production of tartaric acid probably was more sensitive to climatic conditions found in August such as higher average temperature (17.6ºC) and less hours of sunshine (217). Furthermore, the increase in the content of this acid could also (at least partly) explain the decrease of pH observed in samples packaged in film F1 in August.

2.3. Effect of storage time on physicochemical characteristics of packaged and air stored cut iceberg lettuce

Physicochemical characteristics of cut lettuce are influenced by storage time. A PLS-DA model was performed in order to discriminate the physiochemical characteristics through the storage time.

Figure 3. PLS-DA model of physicochemical characteristics and gas composition of cut lettuce stored for up to 11 days in passive MAP F1 and F2 and for 5 days in air at 5 ℃. *Abbreviations: D1, first day of storage; D5, fifth day of storage; D8, eighth day of storage; D11, eleventh day of storage.*

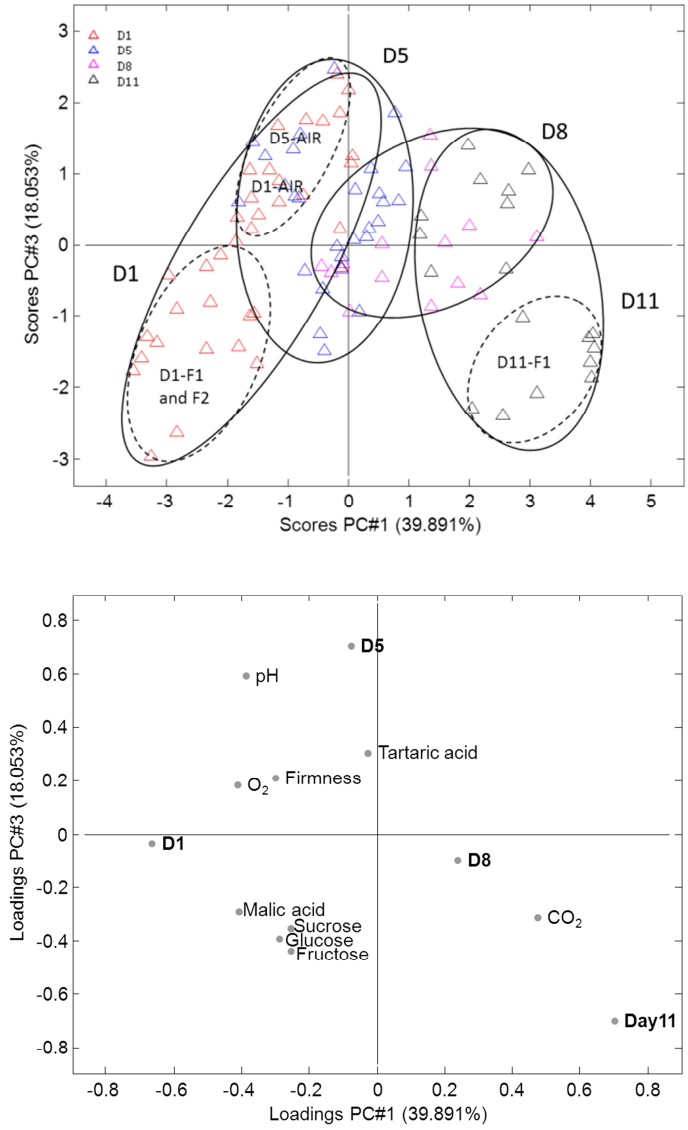
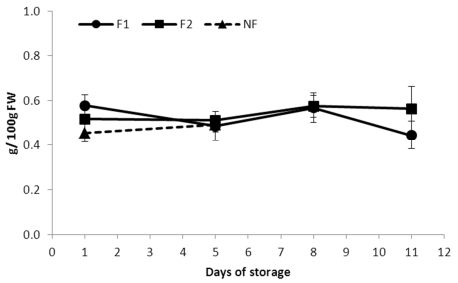
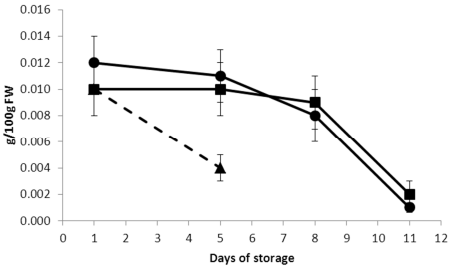


Figure 4. Changes in the concentration of selected soluble sugars, organic acids and firmness of cut lettuce stored for up to 11 days in passive MAP F1 and F2 and for 5 days in air at 5 ºC. Vertical lines represent the standard error of the mean (n=15).

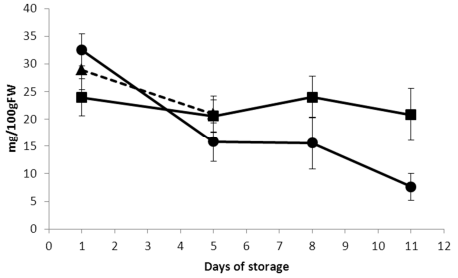
a) Fructose



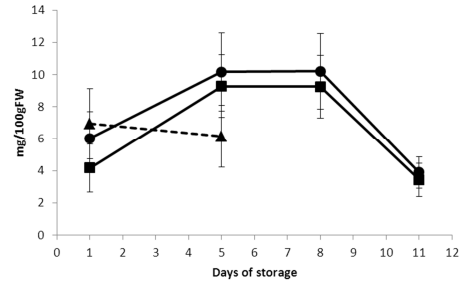
b) Sucrose



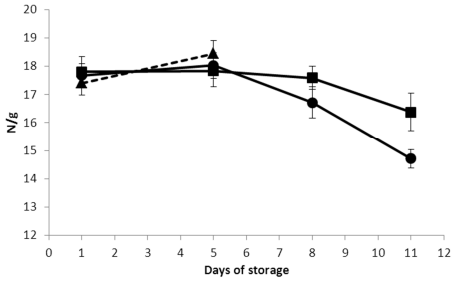
c) Malic acid



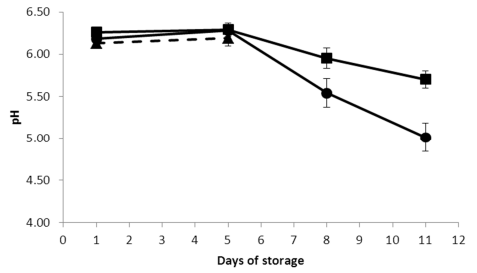
d) Tartaric acid



e) Firmness



f) pH



From the score plot of Fig.3, it can be seen that all the samples were displaced clockwise from day 1 to day 11 of storage. The displacement of samples evidenced that changes on physicochemical characteristics seemed to be related to changes in O_2 and CO_2 as storage time increased. From the loading plot of Fig.3, after 1 day of storage, a high concentration of glucose, fructose, sucrose and malic seemed to be related with packaged cut lettuce (MAP F1 and F2). Firmness and pH tended to be high in air stored samples at 1 and 5 days of storage. Likewise, after 5 and 8 days of storage it seemed there was an increase in tartaric acid in both passive MAPs. After 11 days of storage all physicochemical characteristics except CO_2 tended to decrease probably associated with the extremely high CO_2 content in the packaged samples, mainly in passive MAP F1.

The PLS-DA was confirmed by a 4-way ANOVA. In air stored samples there was a significant drop in sucrose, malic acid and tartaric after 5 days of storage compared to packaged cut lettuce ($p \leq 0.05$), whereas glucose, fructose, firmness and pH were kept constant during the first 5 days of storage. Another trend was observed in packaged cut lettuce. After 11 days of storage there was a significant decrease in firmness and pH in passive MAP F1. For both passive MAPs there was a drop in sucrose after 11 days of storage, but the concentration of fructose and glucose were kept constant ($p \leq 0.05$). Among the acids, malic acid decreased significantly in passive MAP F1 after 11 days of storage ($p \leq 0.05$), but in passive MAP F2 its concentration was kept unchanged during the storage time ($p \geq 0.05$). For tartaric acid a significant increase was found in both passive MAPs after 5 and 8 days of storage ($p \leq 0.05$) (Fig.4).

Glucose and fructose are substrates for the glycolysis, the initial oxidative step in the respiration pathway [21]. The drop in sucrose in packaged and air stored samples was probably owing to its transformation to glucose and fructose by an invertase [22], which could explain that fructose and glucose kept constant during storage. The drop was more substantial in air stored samples in order to meet a high respiratory demanded than under passive MAP. Respiration rate of cut fruits and vegetables is reduced under low O_2 and high CO_2 [23], but extremely low O_2 found in the passive MAPs can trigger anaerobic conditions. Furthermore, malic acid can be used as a respiratory substrate in vegetables and fruits [23], which could explain its extensive loss under air stored conditions. However, the drop in the concentration of malic acid in passive MAP F1 was probably due to malolactic fermentation caused by lactic acid bacteria [24]. Lactic acid bacteria are part of the typical microflora of vegetables, which growth can be enhanced by CO_2 [25]. High content of CO_2 would also explain the drop in pH in MAP F1 after 11 days of storage. The results also indicate that an extensive exposition to high CO_2 after 11 days of storage promoted membrane damage and thereby loss of firmness. Loss of turgor due to damage to cellular membrane has been reported earlier [26]. Hamza *et al*. [27] found that excessive accumulation of CO_2 in packages enhanced tissue softening in packaged romaine lettuce. To our knowledge, the formation pathway of tartaric acid in lettuce is still unknown. In grapes, it has been found that the synthesis of tartaric acid lays outside the TCA and begins with ascorbic acid

[28]. There is no evidence in this study to confirm that ascorbic acids lead to tartaric acid. Therefore, it is suggested that tartaric acid was readily oxided under air stored conditions, whereas its accumulation in both passive MAPs could be consequence of extremely low O_2 and high CO_2.

In general, these results indicate that packaged cut lettuce in passive MAP F2 can delay the loss of sugars, firmness and malic acid, up to 8 days of storage, but longer storage under extremely low O_2 and high CO_2 leads to loss of soluble sugars and firmness as well as this condition stimulates acidification of cut lettuce by an increase in tartaric acid and decrease in pH and development of malolactic fermentation, mainly in passive MAP F1.

2.4.Browning and the relation with chlorogenic acid, ascorbic acid and polyphenoloxydase (PPO) activity - Effect of season, packaging, cultivar and storage time

Browning has been indicated to be the main limitation of the shelf-life of cut lettuce [4]. The potentiality of browning in cut lettuce depends of various factors such us PPO activity, concentration of phenolic acids, natural inhibitors, like ascorbic acids as well as the concentration of O_2 in the package [29]. The relationship between chlorogenic acid, ascorbic acid and PPO activity in the formation of browning in lettuce has been previously studied by Heimdal *et al.* [4] and Degl' Innocenti *et al.* [30]. However, this study provides more realistic information since lettuce was packaged under industrial practices during a whole season.

Browning was not observed in passive MAP F1 and F2 during the storage time. The inhibition of browning was most likely a consequence of extremely low O_2 content and high CO_2 [4, 5]. Low O_2 can reduce the PPO activity in cut lettuce due to O_2 is necessary for PPO to begin the browning in the cut area [29]. In our study PPO activity significantly decreased in cut lettuce packaged in passive MAP F2 after 5 and 8 days of storage ($p \leq 0.05$) (Table 3). PPO activity can also be inhibited by ascorbic acid [30]. It inhibits the activity of PPO by reducing the *o*-quinones back to the *o*-dihydroxyphenol until is totally converted to dehydroascorbic acid [30, 31]. Ascorbic acid was significantly higher after 1 day of storage, but dropped after 5 days of storage as PPO activity (Table 3). Moreover, high CO_2 content could be the cause of a significantly decreased in chlorogenic acid ($p \leq 0.05$) (Table 3), which could reduce the potential of browning [31]. High CO_2 might have caused a decrease in the activity of phenylalanine ammonia-lyase (PAL) by a drop in pH [32]. PAL enzyme leads to the biosynthesis of phenolic acids in cut lettuce [30, 32]. PAL activity was not under study in this paper, but pH decreased in passive MAPs, as explained in section 2.3.

Table 3. Changes in ascorbic acid and chlorogenic acid of cut lettuce packaged in passive MAPs and PPO activity of passive MAP F2 as storage time increase.

Storage time (Days)	Ascorbic acid (mg/gFW)	Chlorogenic acid (mg/gFW)	PPO activity (U ml^{-1})
1	25.28 ± 23.84 (17) ^{b}	53.57 ± 38.17 (17) ^{bc}	0.28 ± 0.10 (11)^{b}
5	7.61 ± 7.26 (20) ^{a}	30.14 ± 45.34 (20) ^{ab}	0.15 ± 0.08 (5)^{a}
8	10.37 ± 10.16 (25) ^{a}	63.49 ± 65.88 (25) ^{bc}	0.16 ± 0.06 (5) ^{a}
11	2.43 ± 3.09 (16) ^{a}	15.33 ± 17.18 (16) ^{a}	

Data expressed as mean±standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$. Abbreviations: FW= fresh weight.

As expected, cut lettuce gradually turned brown after 5 days of storage in air stored samples. Our results indicated that in air stored samples the concentration of chlorogenic acid was significantly higher than in packaged samples *($p \leq 0.05$)* (Table 4) and PPO activity remained constant during the first 5 days of storage *($p \geq 0.05$)* (Table 5). These conditions favored the formation of browning in air stored samples. Baur *et al*. [11] found that PPO activity remained constant when cut lettuce segments were stored under conditions that avoid CO_2 accumulation. In addition, ascorbic acid might have not acted as an effective inhibitor of browning in cut lettuce due to it decreased after 5 days of storage mainly caused by exposition to air. Season and cultivar are important factors for the development of browning [31]. Analysis of variance showed that browning was higher in August and September than in the rest of months *($p \leq 0.05$)* and for cultivar Platinas than Morinas *($p \leq 0.05$)* (Table 6).

Table 4. Concentration of chlorogenic acid in cut lettuce packaged in passive MAPs and stored in air.

	Chlorogenic acid (mg/g FW)
Air	87.21± 58.47 (15) ^{b}
passive MAP F2	48.98 ± 57.16 (14) ^{ab}
passive MAP F1	35.99 ± 32.66 (23) ^{a}

Data expressed as mean±standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$.

Table 5. Changes in PPO activity of cut lettuce stored in air and packaged in passive MAP F2 after 5 days of storage at 5 ºC.

Storage time (Days)	Air ($U\ ml^{-1}$)	passive MAP F2 ($U\ ml^{-1}$)
1	0.19 ± 0.16 (10) [ab]	0.28 ± 0.10 (11) [b]
5	0.11 ± 0.04 (7) [a]	0.15 ± 0.08 (6) [a]

Data expressed as mean±standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$.

Table 6. Brown area fraction (BA) of two cultivars of cut lettuce stored in air at 5 ºC during season 2009.

Factors	BA(%)
Cultivars	
Morinas	9.0 ± 4.5 (8) [a]
Platinas	13.0 ± 4.8 (6) [b]
Season 2009	
June	7.0 ± 1.3 (2) [ab]
July	6.0 ±3.2 (4) [a]
August	12.0 ±0.7 (4) [bc]
September	15.0±5.3 (4) [c]

Data expressed as mean±standard deviation. Values in parentheses represent the number of samples used for the calculation of the mean. Different letters indicate significant differences at $p \leq 0.05$.

3. Materials and Methods

3.1. Plant material and packaging

For this study two commercially cultivars of Iceberg lettuce (*Lactuca sativa* L.), Platinas and Morinas were selected. These cultivars were provided by Rijk Zwaan, Odense, Denmark). The experiment was conducted in the year in 2009. The plant material was sowing in the commercial facilities of Bladgrønt, Denmark and transplanted to an open field in Bogense, Fyn, Denmark once lettuces had 4 to 6 leaves per plant. Details on sowing and transplanting dates can be found in Deza-Durand and Petersen [14]. The harvest of lettuce was carried out on 10th June, 20th July, 18th August and 8th September, respectively. Lettuces were harvested at commercial maturity.

Iceberg lettuces were minimally processed in the facilities of the vegetable processing factory Gasa Odense, Denmark. Each lettuce cultivar was processed separately. Three different packaging treatments were established. Two modified passive atmosphere packaging made of two different films, MAP F1 (OPALEN 65 AF (65 μm)) and MAP F2 (OPP/PE-L 2040 AF (60

μm)). These films are commercially used by the processor. The O_2 and CO_2 transmission rate for film F1 was 35 and 158 cm^3 m^{-2} 24 h^{-1} atm^{-1} at 23 ºC and 50% RH, respectively. For film F2 the O_2 transmission rate was 68 cm^3 m^{-2} 24 h^{-1} atm^{-1} at 23 ºC and 85% RH, but no data was provided for CO_2 transmission rate by the manufacturer Bemis Packaging (Horsens, Denmark). In the laboratory, a third treatment consisting of cut lettuce stored in air was set up. An air treatment was chosen with the finality to characterize the difference in physicochemical changes against packaged lettuce. Details of set up of this treatment are described in Deza-Durand and Petersen [14]. Samples of passive MAPs were stored in duplicates for up to 11 days, whereas, air stored samples were only kept for 5 days due to browning. All samples weighed 250 g and were stored at 5 °C in cool chambers in the laboratory (Termaks AS, Norway).

3.2. Analysis of sugars, organic acids and chlorogenic acid by gas chromatography-mass spectrometry (GC-MS)

3.2.1. Freeze dried lettuce

Fifty grams of each cultivar packaged in passive MAP F1 and MAP F2 and stored in air were freeze dried and kept at -80 ºC. This was performed after 1, 5, 8 and 11 days of storage for packaged samples and after 1 and 5 days of storage for air stored samples. The samples were then fine-ground and stored at -20 °C in airtight bags until analyses.

3.2.2. Derivatization of glucose, fructose, sucrose, malic acid and tartaric acid

Seventy five milligrams of freeze dried powder lettuce were weighted and placed in a centrifuge tube and extracted with 3 mL of Millq water in an ice bath with stirring 800 rpm for 1 h. After the extraction, the samples were centrifuged at 3000 rpm for 20 min at 2 °C (Sorvall RT 6000, DuPont Company, Delaware, USA). The supernatant was filtered (0.45 μm) and 5 μL of the filtrate was placed in a vial and evaporated to dryness at 80 ºC. Derivatization was performed by adding 100 μL of the silylation mixture Fluka I to the vial (Sigma-Aldrich, Denmark AS). The vial was closed, shaken with a vortex for 3 min, and incubated at 60 ºC for 30 min.

3.2.3. Derivatization of chlorogenic acid and ascorbic acid

Ten milligrams of freeze dried powder lettuce were weighted and placed in a 2 mL glass tube and added 600 μL of the same derivatization reagent as above. Then the tube was shaken by a vortex for 3 min. The tube was incubated at 60 ºC for 30 min and left at room temperature overnight. After that, the tube was centrifuged at 2000 rpm for 5 min.

3.3. Gas chromatography (GC)-Mass spectrometry (MS)

The trimethylsilyl (TMS) derivatives of soluble sugar, organic acids, chlorogenic acid and ascorbic acid were separated and identified on an Agilent GC-MS system with an autosampler. Two microliter was injected into the GC-MS using a split ratio of 1:10 for derivatives of chlorogenic acid, ascorbic acid and sucrose. A split ratio of 1:200 was applied during injection of the derivatives of glucose, fructose, malic acid and tartaric acid. The GC was equipped with a nonpolar column ZB-PAAC CGO-7169 (15 m x 0.25 mm x 0.25 μm) (Phenomenex Inc., USA). The column flow rate was 1.5 mL min^{-1} using helium as carrier gas. The temperature of the column was held at 90 °C for 1 min and then increased by 6 °C min^{-1} up to 200 °C, which was kept constant for 1 min, then increased by 20 ºC min^{-1} up to 320 ºC and then held for 10 min. The mass spectrometric detector operated in electron ionization mode and scanned mass/charge (m/z) between 20 and 500. All TMS derivatives identification was made by matching obtained mass spectra with those in the G1035A Wiley library (Hewlett-Packard, Palo Alto, CA, USA) and by comparison of the retention time and mass spectra with those of authentic reference standards.

Standard curves were prepared for quantitative estimation of glucose, fructose, sucrose, tartaric acid, ascorbic acid and chlorogenic acid. The concentration of glucose, fructose and sucrose was expressed in g 100 g^{-1} of fresh weight (FW) and those from tartaric acid, ascorbic acid and chlorogenic acid were expressed in mg 100 g^{-1} of fresh weight (FW).

3.4. Polyphenol oxidase(PPO) activity

PPO activity was only performed in crude extracts of air stored samples after 5 days of storage and in passive MAP F2 after 8 days of storage. The latter was chosen as an example since no browning was observed in any of the passive MAP packages.

PPO was extracted according to the method of Heimdal *et al.*[4] with some modifications. Seventy five milligrams of freeze dried powdered lettuce were weighted and placed in a 50 mL centrifuge tube and extracted with 15 mL of water in an ice bath with stirring at 700 rpm for 2 h. After the extraction, the samples were centrifuged at 3000 rpm for 20 min at 2 °C (Sorvall RT 6000, DuPont Company, Delaware, USA). The supernatant was filtered with Muncktell 00H 9cm paper in an ice bath. Finally, the filtrate were transferred into 50 mL centrifuge tube and stored in an ice bath before the assay. The crude extract was stored for no more than 1 day.

PPO activity was assayed spectrophotometrically by measuring the depletion of chlorogenic acid at 326 nm in a Shimadzu 1601 spectrophotometer (Shimadzu Corporation, USA). The reaction mixture was 1 mL 0.1mM chlorogenic acid in 20mM phosphate buffer (pH 7.0), 1.45 mL of 200mM phosphate buffer (pH 7.0), and 50 μL of the enzyme extract, respectively, with a final volume of 2.50 mL. The assay was performed for 1 min. A blind test on the substrate and enzyme was performed.

PPO activity was expressed in U ml^{-1} using the following equation [33]:

v_o/ enzyme volume= $(a/\varepsilon_{\lambda max}\ lb10^{-3})*(-\Delta A_{\lambda max}/\Delta t)$

where a=total assay volume (mL), b=enzyme volume, $\varepsilon_{\lambda max}$=molar extinction coefficient of chlorogenic acid (20000 M^{-1} cm^{-1}), l=path length of the cuvette (cm) and $\Delta A_{\lambda max}/\Delta t$ =change in absorbance as function of time. The minus sign in the equation makes the change in absorbance positive as chlorogenic acid is decreasing during the reaction. The assay was performed in duplicates.

3.5. pH

Twenty five grams of cut lettuce from packaged samples and air stored samples were frozen at -20 ºC after 1, 5, 8 and 11 days and 1 and 5 days, respectively. To determine the pH the samples were defrosted and allowed to reach room temperature before the electrode was placed inside the plastic bag that contained the sample. The pH measurement was performed using a portable pH meter (Portamess 913 pH, Knick, Germany) previously calibrated with buffer solutions of pH 4 and 7.

3.6. Colour measurements

3.6.1. Image acquisition

Images of cut lettuce from each film package and air stored samples were captured using a desktop flatbed scanner after 1, 5, 8 and 11 days of storage for packaged samples and after 1 and 5 days of storage for air stored. Images included a colour reference consisting of 20 colour patches made of dyes used for painting. The scanner was covered with a black lid in order to exclude surrounding light. All images were saved in an uncompressed format (TIFF).

3.6.2. Image analysis

For colour correction a transformation was sought to each image to bring the colours of the reference patches to match a reference image. Details of colour correction and thresholding are found in Deza-Durand *et al*. [34]. After thresholding, the brown area fraction (BA) was calculated as follows:

Brown area fraction= # brown pixels /#total pixels

The ImageJ software was used for colour correction and thresholding of the images [35].

3.7. Firmness

Texture measurements were performed using a TA-XT plus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK) with a Krammer shear press with five blades. All tests were performed with 15 g of cut lettuce. The texture was expressed as firmness that was defined as the maximum peak force during the shear compression.

3.8. Statistical Analysis

Partial least square discriminant analysis (PLS-DA) was carried out for discrimination of fructose, glucose, sucrose, firmness, pH, malic acid, tartaric acid O_2 and CO_2 levels among season and storage time. PLS-DA models were fully cross validated. Principal component analysis (PCA) was chosen for the description of the main variation in complex data of browning, chlorogenic acid, ascorbic acid and gas composition of packaged and air stored samples.

The effect of season (S), cultivar (C), packaging (P) and storage time (T) on soluble sugars, organic acids, firmness and pH was evaluated by a four-way ANOVA and also by one-way ANOVA for each main factor for chlorogenic acid, ascorbic acid and PPO activity. The samples were analyzed in two sets in order to avoid problems with unbalance in the interaction P x T, because air stored samples were only analyzed at 1 and 5 days of storage. In the first set, the three main factors (C, S, P) with the respective levels were analyzed at 1 and at 5 days of storage. In the second set, films F1 and F2 were analyzed together with levels of S, C and T. Browning was only observed in air stored samples after 5 days of storage, therefore two-way ANOVA for cultivar and season was performed on this data. The variations in O_2 and CO_2 in passive MAPs were also subjected to four-way ANOVA.

Significance of difference between means was determined by Tukey's multiple range test at 95% confidence. Means and ANOVA were performed with Infostat Statistical Software [36], whereas PLS-DA was carried out using Latentix Ver. 2.00 (Latent5, Copenhagen, Denmark, www.latentix.com).

4. Conclusions

The aim of this study was to assess the influence of season, cultivar, packaging and storage time on physicochemical characteristics of cut iceberg lettuce. Season, packaging and storage time mainly influence over physicochemical characteristics of lettuce, and in less degree cultivar. In June soluble sugars, malic acid and firmness were kept high, which indicate that probably will be easier to maintain a better quality product in June than the rest of months.

It is concluded that passive modified atmosphere is appropriated for storage cut lettuce, contrary as previous research recommended. Our data evidenced that by the use of film F2 the recommended limit of O_2 for packaged cut lettuce (1%) can be further reduced to 0.1-0.3% O_2 and the higher limit of CO_2 can be set to 22% at 5 ºC due to no browning and minimum loss of sugars, firmness and malic acid was found for up to 8 days. However, longer the storage favored tissue softening, decreased of sugars, acidification and malolactic fermentation, as found mainly in passive MAP F1. Therefore, storage packaged cut lettuce for up to 11 days cannot be recommended. Sensory evaluations are needed in order to identify passive MAP F2 as promising for packaged cut lettuce.

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